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INVESTIGATION OF THE VARIABLES IN THE FECAL UROBILINOGEN PROCEDURE WITH A PROPOSED MODIFICATION *

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INTRODUCTION

For over twenty years the determination of fecal urobilinogen has been of value in the diagnosis of hepatic and hemolytic diseases. The accepted method has been that developed by Watson, et al, based on Ehrlich's reaction with aldehydes.[3,5,6,7] Since the Ehrlich's reaction is not specific for urobilinogen, an extraction with petroleum ether is included in the procedure in order to avoid measuring compounds other than urobilinogen.

In this hospital the determination was performed in a research department only by a few experienced workers until two years ago. At that time the routine clinical laboratory started using the method. It was soon apparent that there were many steps in the procedure that were being interpreted in different ways by the many staff persons and students that used the test. Widely discrepant results were obtained. The questions of preservation of specimens and timing at various steps of the procedure also became more important when the method was moved to the routine clinical laboratory where the tests were done in batches and other procedures were being performed simultaneously. The results of the investigation of these technical points are presented in this paper.

While an analysis of the variables of the fecal urobilinogen method was being made, it became apparent that the lengthy extraction procedure did not lend itself to multiple determinations. A modified extraction technique was developed that reduced the number of extractions and employed a mechanical shaking device. This modified method is described and the results obtained with it are discussed.

EXPERIMENTAL

Method

The method described by Watson, et al, both for the Ehrlich's reaction directly on ferrous hydroxide filtrate and on petroleum ether extracted urobilinogen, has been used unless otherwise indicated.[3,5,6,7] The term urobilinogen in this paper will refer to the group of compounds that

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includes stereobilirubinogen and mesobilirubinogen and which give identical color reactions with Ehrlich's reagent. The term urobilin will refer to the oxidized forms of the compounds just mentioned. Reports in Ehrlich Units will imply that filtrate from a ferrous hydroxide mixture of feces has been combined directly with Ehrlich's reagent and sodium acetate, and represents color from urobilinogen as well as other aldehyde reacting compounds. Results in milligrams will imply that only petroleum ether soluble compounds have been measured.

Reagents

1. Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)
   20 grams plus 92 ml. deionized water, prepared just before addition to diluted feces.
2. Sodium hydroxide, 10%
3. Petroleum Ether
4. Glacial Acetic Acid
5. Ehrlich's Reagent
   0.7 grams para-dimethyl-amino-benzaldehyde
   150 ml. concentrated hydrochloric acid
   100 ml.deionized water
6. Sodium acetate, saturated, reagent grade

Preservation of Specimens

An Ehrlich's reaction was performed directly on the filtrates of ferrous hydroxide-stool mixtures set up before and after four days stool collections were frozen at —10 degrees Centigrade. The specimens were frozen for various periods of time in cardboard collection cartons. The changes after storage are noted in Table I.

TABLE I

Preservation of fecal specimens by freezing

Specimen	Ehrlich Units Percent Days After Collection of Specimen 0	3	6	9	12	15	18	21	24	27	30	Increase or Decrease After Storage
1	53			63								+19%
2	73			83							83	+14
3	212									230		+ 8
4	44									47		+ 7
5	201			191								— 4
6	159			200								+26
7	193			222								+15
8	1380				1051							—24
9	863				908							+ 5
10	808	715	648*									—14* —20

Average change after freezing + 4%

* Same specimen kept at room temperature.

Time for Reduction of Urobilin

Ferrous hydroxide mixtures with four day stools were set up and placed in a dark place. At various time intervals after the feces and reagents were combined, the mixtures were filtered and an Ehrlich's reaction was carried out on the filtrate. Table II shows results obtained

over the first three hours of reduction; Table III presents results obtained over a twenty-four hour period. Specimens 1, 2 and 3 are the same stool specimens on both tables.

TABLE II

Ehrlich reactions on filtrates separated from ferrous hydroxide mixture after 15 to 170 minutes of reduction time.

Specimen	Ehrlich Units Percent Minutes After Combination of Feces with Hydroxide 15	30	45	60	75	90	105	120	135	150	165	180
1		128	124			130	135		133			
2		442		486		505		471				
3		195	192		205		214				216	
4	332		405		408		437				448	

TABLE III

Ehrlich reactions on filtrate separated from ferrous hydroxide mixture after 1 to 24 hours of reduction.

Specimen	Ehrlich Unit Percent Hours After Combination of Feces with Ferrous Hydroxide 2	4	6	8	10	12	14	16	20	22	24	First-Last Analysis x 100 First Analysis
1	130	136							139			+ 7%
2	486	471							488			— 3
3	214	216							224			+ 9
4	1030								1010			— 2
5								224			219	— 2
6	965								950			— 2
7	980								959			— 2
8		96					89					— 7
9		119							107			—10
10		179							181			+ 1
11									521		500	— 4
12									525		489	— 7
13									340		312	— 8

Five specimens were also extracted after various reduction times; the modified extraction technique described below was used. The results of these extractions appear in Table IV with corresponding values obtained with the direct Ehrlich reaction on the same filtrates.

TABLE IV

Extraction of filtrates separated from ferrous hydroxide mixtures after various time periods.

A = Milligrams Percent Urobilinogen by Extraction
B = Ehrlich Units Percent by Direct Reaction on Filtrate

	Hours After Combination of Feces and Ferrous Hydroxide That Mixture Was Filtered 4		15		17		21		42		First-Second x 100 First	
Specimen	A	B	A	B	A	B	A	B	A	B	A	B
1	98	98	66	89	..	..	..	..	..	..	—33%	—0%
2	85	119	..	..	..	..	68	107	..	..	—20	—10
3	142	179	..	..	..	..	126	181	..	..	—11	+ 1
4	..	..	..	..	740	965	..	..	612	..	—17	....
										Average Change	—20%	— 6%

Blanks and Color Development

In the procedure described by Watson, an unknown blank is prepared by reversing the order of the reagents added to filtrate[3], the sodium acetate neutralizes the hydrochloric acid in Ehrlich's reagent as it is added and no color development takes place. Made in this way, the blanks for some specimens high in urobilinogen concentration develop some color. This difficulty can be avoided by mixing the Ehrlich's reagent and sodium acetate, waiting about 30 seconds and then adding the filtrate. Blanks for any unknowns that require dilution of color development are made by diluting the blank to the same volumes as the developed color, but with the Ehrlich's reagent and sodium acetate combined. Color development always occurs in the blanks if the sodium acetate is not saturated.

Watson, et al, state that maximum color is developed when the ratio of Ehrlich's reagent to saturated sodium acetate is 1:2 or 1:3;[3] this point has been verified in this laboratory.

Frequently the color developed in an Ehrlich's reaction is too intense to measure in a colorimeter. The usual method of dilution with water has been compared with three other dilution methods, all of which maintain the optimal ratio of Ehrlich's reagent to acetate. The four methods compared are:

1. Make an appropriate dilution of filtrate with deionized water. Combine one part of this dilution with one part Ehrlich's reagent, mix and add two parts saturated sodium acetate.
2. Make an appropriate dilution of filtrate with a "blank filtrate" (water, ferrous sulfate and hydroxide in the same proportions as the feces mixture). Combine an aliquot of this dilution with one part Ehrlich's and two parts of saturated sodium acetate.
3. Combine one part filtrate with one of Ehrlich's reagent, mix and add two parts saturated sodium acetate. Dilute this mixture with water.
4. Use less filtrate, maintain the proportions of reagents, e.g. add 1 ml. filtrate to 4 ml. Ehrlich's reagent, mix and add 8 ml. acetate. If color is still too intense, combine an aliquot of the filtrate-Ehrlich's-acetate mixture with an equal amount of Ehrlich's reagent, mix and add two parts saturated sodium acetate.

Table V shows a number of comparisons of the results of direct Ehrlich reactions obtained on filtrates when more than one of the four dilution methods were used. Columns 1 through 4 correspond to methods 1 through 4 above. The dilution volume listed by each result applies to the dilution of filtrate with water or "blank filtrate" before addition of reagents in methods 1 and 2, to the dilution of combined filtrate and reagents with water in method 3, and to the final dilution of filtrate with reagents in method 4.

Stability of Color Development

It has been reported that with reagent grade sodium acetate, color development remains stable at least 70 minutes.[2] Reagent grade sodium acetate is used in this laboratory but a loss of color has been observed.

Readings are taken within five minutes of their color development, both with the direct Ehrlich's reaction on filtrate and after extraction.

TABLE V

Comparison of results from four dilution methods used with the direct Ehrlich's reaction on 18 filtrates.

Specimen	DILUTION METHOD							
	1		2		3		4	
	Diln.	E.U. %	Diln.	E.U. %	Diln.	E.U. %	Diln.	E.U. %
1	1:20 1:25 1:30	845 880 925	1:20 1:25 1:30	795 820 885	1:20 1:25 1:30	720 692 673	1:80 1:100 1:120	1010 1063 1030
2	1:4 1:6	134 135	1:4 1:6	138 133	1:4 1:6	111 113	1:15 1:60	139 149
3	....	....	1:20	659	....	....	1:72	806
4	....	....	1:20	784	....	....	1:72	950
5	....	....	1:20	973	....	....	1:72	1170
6	....	....	1:20	909	....	....	1:72	959
7	1:6 1:10	160 157	....	....	1:6	128	....	....
8	1:10	197	....	....	1:6	175	....	....
9	1:6 1:10	202 200	....	....	1:6	171	....	....
10	1:10 1:15 1:20	510 503 470	....	....	1:4 1:8 1:10	388 395 452	....	....
11	....	....	....	151	....	149	....	....
12	....	....	....	219	....	140	....	....
13	1:10	248	....	....	1:10	235	....	....
14	1:10	235	....	....	1:10	213	....	....
15	1:20	448	....	....	1:20	394	....	....
16	1:10	246	....	....	1:10	216	....	....
17	1:20	1058	....	....	....	....	1:144	1380
18	1:10 1:20	666 666	....	....	....	....	1:144	863

Extraction with Petroleum Ether

Extractions were performed on the filtrates of nine specimens with and without washing the pooled petroleum ether with water. The results appear in Table VI. Deionized water was used for washing all specimens except numbers eight and nine; distilled water was used in those extractions. In most cases, a single wash with 10 ml. of water was used but two and three washes were used in a few cases and these are indicated.

A series of extractions were made on eight specimens with and without adding water to the filtrate before shaking it with petroleum ether. Seven of these comparisons were made during the time when the laboratory used distilled water. The laboratory now uses deionized water and

TABLE VI

Extraction with and without the Water Wash of Petroleum Ether

SPECIMEN	Milligrams Percent Wash Omitted	Milligrams Percent Combined Petroleum Ether Extracts Washed Once With 10 ml. of Deionized Water
1	17	12, 12
2	189	157
3	446	438
4	438	384
5	424	414*
6	446	446*
7	768	658,* 778
8‡	...	144, †202, 216, 201
9‡	38	22,* 26

* Two washes with 10 ml. water.
† Three washes with 10 ml. water.
‡ distilled water.

this was used in the extraction of specimen 8 in Table VII. Some of the extraction results are higher than the corresponding values from the direct Ehrlich's reaction on filtrate because the color from extractions was diluted with reagents while the direct reactions were diluted with water.

TABLE VII

Extraction with and without addition of water to filtrate

SPECIMEN	Uhrlich Units Percent	MILLIGRAMS PERCENT UROBILINOGEN	
		Filtrate Diluted to 50 Ml. with Distilled Water	Water Omitted
1	73, 83, 83	10, 9, 10	68
2	212, 230	19	260
3	666	37, 260	689
4	44, 47	5	20
5	66	3	48
6	735	167, 252, 260	670
7	222	127	201, 202, 216, 144
8	965	713*	778

* Deionized.

As with the Ehrlich's reaction on filtrate, the color developed after extraction sometimes needs dilution. Dilution with deionized water was compared with dilution with reagents on six specimens. The results appear in Table VIII with dilution factors; e.g. a 1:4 dilution factor means that one volume of the developed color was diluted to four volumes, either with water or reagents.

TABLE VIII

Dilution of color after extraction

SPECIMEN	Dilution with Water		Dilution with Ehrlich's Reagent and Sodium Acetate	
	Mg. Percent	Dilution	Mg. Percent	Dilution
1	395	1:8	453	1:8
2	82	1:4	88	1:4
3	137	1:4	144	1:4
4	76	1:4	78	1:4
5	120	1:4	127	1:4
6	136	1:4	144	1:4

Modification of Extraction

The extraction technique was modified in order to avoid several of its unattractive features: the need for transferring filtrate in and out of the separatory funnel several times with consequent small losses in volume and exposure to oxidation, and the time consuming multiple extractions (three with petroleum ether, one or two with wash, and two to six with Ehrlich's reagent). The modifications were these:

1. Filtrate was measured into a 125 ml. reagent bottle. No water was added. 50 ml. of petroleum ether and glacial acetic acid were added and the extraction was carried out on a Burrel wrist action shaker with a one inch excursion and approximately 200 oscillations per minute. The amount of filtrate and acid and the shaking time are discussed below.
2. The petroleum ether was not washed.
3. An aliquot of the petroleum ether layer was transferred to a 50 ml. reagent bottle. Ehrlich's reagent was added and the two were mixed on the automatic shaker. The volumes of the two were varied as discussed below.
4. Sodium acetate was added to the contents of the reagent bottle, the bottle was shaken to mix the contents well and then emptied into a colorimeter tube. The absorption was immediately measured with an Evelyn colorimeter.

Using this modified technique, multiple extractions were carried out in an attempt to define optimal conditions for single extractions of urobilinogen.

To determine the best shaking time for both the petroleum ether and Ehrlich's extractions, 5 ml. aliquots of filtrate plus 0.5 ml. glacial acetic acid were shaken with 50 ml. of petroleum ether for different periods of time. 5 ml. aliquots of the petroleum ether layers were then shaken with 5 ml. of Ehrlich's reagent for timed periods. Color was developed with sodium acetate and read at once on a colorimeter. The results appear in Table IX.

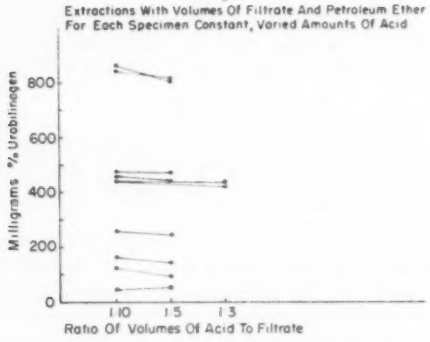
TABLE IX

Determination of Optimal shaking times for single extractions with petroleum ether and Ehrlich's reagent

SPECIMEN	Minutes Shaken with Ehrlich's Reagent	MILLIGRAMS PERCENT UROBILINOGEN Minutes Shaken with Petroleum Ether					
		1	3	5	10	15	20
1	1	471	...	...	538	...	...
2	1	412	...	...	456	...	...
3	1	540	555	624	585	...	...
	5	578	612	629	605	...	...
	10	619	639	655	612	...	...
4	1	...	...	238	...	...	...
	2	...	...	244	244	244	244
	5	...	...	252	257	252	252
	10	...	...	256	254	256	258
	15	...	...	...	254	256	250

Using the modified extraction technique described above, extractions were made of ten specimens using ratios of acid to filtrate of 1:10, 1:5 and 1:3; while the volume of filtrate was the same, the amounts of acid were varied. The altered pH, as shown in Figure 1, did not have the magnitude of effect that was expected. However, when the volume of filtrate from eight different specimens was varied and the concentration of acid was kept constant, much greater discrepancies were observed.

Fig 1



See Figure 2. Only when the ratio of acid to filtrate was kept constant, 1:10, did extractions of various amounts of filtrate give comparable results. Figure 3 shows results of two to three extractions on each of twelve specimens when this ratio of acid to filtrate was maintained. Lower results were obtained when 10 ml. of filtrate were extracted as compared to extractions of 5 ml. on two specimens, even when the 1:10 ratio of acid to filtrate was maintained. This was apparently due to the influence of other substances in the specimens rather than to insufficient

Fig 2

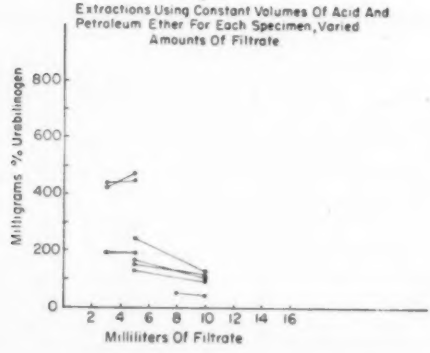
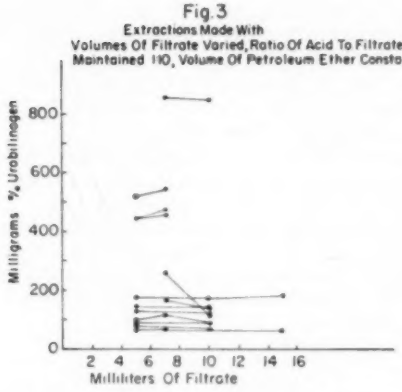


Fig. 3



petroleum ether for extraction because specimens as highly concentrated as 800 milligrams % urobilinogen have been extracted and the results from 5 and 10 ml. filtrate extracted into 50 ml. petroleum ether have been in agreement.

Volumes for the extraction of petroleum ether with Ehrlich's have been varied depending upon the concentration of urobilinogen. 5 ml. of Ehrlich's reagent has been shown to be sufficient to extract all the urobilinogen from very high concentrations: 5 ml. of petroleum ether extracted with 5 ml. of Ehrlich's reagent gave a result of 805 milligrams %, extracted with 10 ml. of Ehrlich's reagent, a result of 795 milligrams %. Second extractions have given negative results.

PROPOSED MODIFICATION

Procedure

1. Weigh and mix the complete four day collection of stool.
2. Weigh 20 grams ferrous sulfate and dissolve in 92 ml. deionized water in a 500 ml. Erlenmeyer flask. This should be done just before adding the stool.
3. Weigh 10 grams of the well mixed stool in an evaporating dish.
4. Grind the stool with portions of 90 ml. deionized water, decanting the supernate after each portion into the ferrous sulfate solution.
5. If the stool is normally dark in color, add another 100 ml. of deionized water. If the stool is acholic, omit this step.
6. Add 100 ml. of 10% sodium hydroxide to the ferrous sulfate-stool mixture with mixing.
7. Put the ferrous sulfate-hydroxide-stool mixture in the dark for at least 2 hours. The remainder of the test may be carried out from 2 to 24 hours after this mixture is made (preferably in the early part of this period).
8. Filter the mixture and immediately transfer 5 ml. of the filtrate to a 125 ml. reagent bottle. Pipette 50 ml. petroleum ether (a 50 ml.

volumeteric pipette is convenient) and 0.5 ml. glacial acetic acid into the bottle. Stopper the bottle and shake on an automatic shaker for 5 to 10 minutes.

9. Transfer 5 ml. of the petroleum ether (upper layer) to a 50 ml. reagent bottle, add 5 ml. Ehrlich's reagent and shake again with the automatic shaker for 10 minutes.
10. Add 10 ml. saturated sodium acetate to the Ehrlich's reagent and shake the bottle to mix. The petroleum ether need not be removed. Pour the mixture into a colorimeter tube and measure the color immediately. If it needs dilution, dilute with appropriate volumes of reagents; e.g. 1 ml. colored solution, 3 ml. Ehrlich's reagent, and 6 ml. saturated sodium acetate. If the colorimeter reading is greater than 90, repeat the extraction using 10 ml. of filtrate and 1 ml. of glacial acetic acid.
11. Calculation:

$$\text{mg/100 ml. from graph} \times \frac{400 \text{ ml.}}{10 \text{ gm.}} \times \frac{50 \text{ ml.}}{5 \text{ ml.}} \times \frac{15 \text{ ml.}}{5 \text{ ml.}} \times \text{any additional dilution factors} = \text{mg./100 gm.}$$

RESULTS

The procedure for extraction with modifications indicated above has been used in this laboratory for three months. Students and staff members trying the procedure for the first time have performed duplicate extractions on fourteen ferrous hydroxide-stool filtrates. Their results, as shown in Table X, indicate an acceptable degree of precision.

TABLE X

Results of Duplicate Extractions on 14 filtrates using the modified extraction method

SPECIMEN	Ehrlich Units Percent	MILLIGRAMS PERCENT UROBILINOGEN	
		1	2
1	52	38	38
2	214	162	168
3	114	84	94
4	87	73	73
5	195	240, 245	248, 248
6	364	360	357
7	320	286	288
8	370	378	363
9	99	74	76
10	205	159	161
11	70	54	52
12	108	86	83
13	157	132	133
14	525	512	509

The results in Table X also demonstrate the relationship between the total aldehyde reactive compounds in a specimen and the amount that is extracted with the modified technique. This relationship is illustrated in a different way for 30 specimens in Figure 4. Five comparisons are made of results from the original and the modified extraction procedures on the same specimens in Table XI.

Fig 4

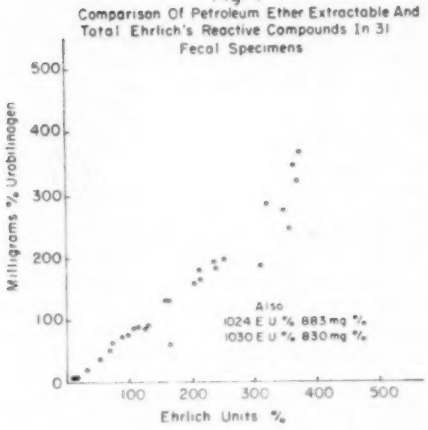


TABLE XI

Comparisons of results obtained with the Watson extraction technique and the modified extraction technique

Ehrlich Units Percent	Original Extraction Technique	Modified Extraction Technique
142	96	136
182	121	157
315	252	259
965	713	740
1010	834	853

DISCUSSION

Although immediate analysis is always desirable, the results in Table I indicate that small changes occur in fecal urobilinogen if the specimens are frozen. Exceptions to this may be specimens of very high concentration; both specimens 8 and 10 showed significant loss during storage. When specimen 10 was stored at room temperature there was a greater loss than after storage at freezing temperatures.

One hour has been suggested as the time necessary for the ferrous hydroxide to reduce the urobilin in a specimen.[5] This would depend upon the age of the specimen as well as its concentration of urobilin since oxidation starts as soon as a specimen is exposed to air. It is important that the ferrous hydroxide mixtures be placed in the dark because light hastens the oxidation of urobilinogen. The results of direct Ehrlich reactions obtained during the first three hours of reduction, as shown in Table II, indicate that a minimum reduction time of two hours might apply safely to more specimens. Results in Table III indicate that reoxidation is slight in the first twenty-four hours of exposure to ferrous hydroxide.

Four specimens that were extracted after various reduction times showed more significant losses with time than did the corresponding direct Ehrlich

reactions. Table IV. These results would indicate that the time of exposure to ferrous hydroxide is more critical than has been suspected when extractions are to be performed. Until further investigation of this point can be made, extractions in this laboratory are performed as soon as possible after the minimum reduction time of 2 hours is passed.

Depending upon the dilution method used, values can be obtained on the same filtrate that differ by as much as 30%. It is apparent that, of the four methods described, method 3 gives the lowest results. This is the method most commonly used, that is, dilution of developed color with water. The two methods that agree best with each other are 1 and 2. Both involve dilution of filtrate before its addition to the Ehrlich's reagent. Method 2 best simulates the conditions of an undiluted Ehrlich's reaction because the alkalinity of undiluted filtrate has been supplied by diluting with "Blank filtrate". The highest results are obtained with method 4. Perhaps the increased acidity intensifies color without relation to aldehyde concentrations present. This might also account for the results from method 1 being higher than those from method 2. If a low pH is required for maximal color development, the water or "blank filtrate" would account for a neutralizing affect on the mixture and also for the lower results obtained with methods 1, 2, and 3. There is also the possibility that method 3 does not provide enough para-dimethyl-amino-benzaldehyde to react with all the urobilinogen in a specimen; this would account for lower results than with the other methods. When dealing with such a nonspecific reaction as the Ehrlich's reaction there is always a question of what is being measured, and how extraneous compounds are affected by the conditions altered when diluting. Furthermore, impurities in water could cause the oxidation of urobilinogen and account for lower results. While investigations are continued regarding this question of dilutions, an emperical choice of method 4 has been made for use with our routine determinations.

It is apparent from the results in Table VI that the washing of petroleum ether with water removes Ehrlich's positive compounds. Whether urobilinogen is removed along with extraneous substances will have to be examined with urobilinogen recovery studies. These results do show that varied numbers of washes, and presumably different volumes of washes, remove varied amounts of reactive material. Consequently, both the volume and the number of washes should be evaluated and then standardized.

When the same filtrates were extracted with and without water added to the filtrate, much lower values were obtained with the extractions that included the water. The exception in Table VII is specimen 8, which was extracted using deionized water. The low values were compared with direct Ehrlich's reactions on the same filtrates and the usual relationship between the extraction and direct values was not demonstrated in these specimens. All other reagents and the details of the technique were carefully examined at that time; one explanation for the low results would be contamination of water. This laboratory has not had similar difficulties with low results when using the Watson method in the period that it has been using deionized water. In the modified technique described, no water is added to the filtrate before extraction.

Of the six comparisons of dilution methods after extraction, Table VIII, only one demonstrates any significant difference between diluting with water or diluting with reagents. In this case, the reagent dilution gave 15% more color than did dilution with water. In the modified technique, dilutions are made with reagents and read against a blank composed of one part Ehrlich's reagent and two parts sodium acetate.

Balikov reported that a single extraction with 35 ml. of petroleum ether shaken for 7 minutes was as efficient as three extractions, each shaken for one minute.[2] In developing the modified technique described above, it was found that the time for shaking both the petroleum ether and the Ehrlich's reagent extractions was critical. The results in Table IX indicate that a single extraction with 50 ml. of petroleum ether should be shaken for at least 5 minutes and that the aliquot of ether should be shaken with Ehrlich's reagent for 10 minutes. These times are for shaking with the automatic device described and would have to be reevaluated for hand shaking. Completeness of the single extraction has been checked with second extractions that gave negative results.

A careful adjustment of pH with glacial acetic acid has been demonstrated to be of importance for the completeness of extraction of urobilinogen.[1] Figures 1, 2 and 3 illustrate the importance of acid concentration in the modified extraction technique. The affect of varying the amount of acid used was not too apparent when a constant volume of 5 or 10 ml. of alkaline filtrate was extracted. When one milliliter of acid was used with various amounts of filtrate, values were more markedly changed. This may present an additional problem when mixtures are made of alcoholic stools and, in reducing the amount of water used for the mixture, it is made more alkaline than the usual mixture. When different amounts of filtrate are extracted after the acidification of the extraction mixture with amounts of glacial acetic acid varied in the proportion of one part acid for every ten parts filtrate, all extraction values agreed. This agreement of different aliquots and a comparison of these results with direct reactions on the same filtrates are helpful in judging whether or not extractions are complete.

It has been suggested that it is important that the Ehrlich's reagent plus sodium acetate be shaken with petroleum ether in order to redissolve indole in the petroleum ether. If this is not done, the indole will be measured as urobilinogen.[4]

The elimination of the wash and the dilution with reagents probably accounts for the slightly higher results obtained with the modified extraction technique when compared to results obtained with the original Watson method. That the modified version is exceptionally reproducible is shown by the results in Table X. A linear relationship exists in most specimens between the total Ehrlich's positive compounds in a ferrous hydroxide filtrate and the petroleum ether extractable portion as shown in Figure 4.

The modified version of the extraction technique has not only proved to be reproducible but it has proved adaptable to performance in batches. A technologist familiar with both techniques can do four determinations in the time that was formerly required for one.

SUMMARY

An investigation of certain variables in the fecal urobilinogen method have been reported. Recommendations are made in regard to storage of specimens and preparation of blanks. Stability of the urobilinogen at various stages of the procedure have been examined.

In developing a shorter extraction method, considerations have been made of the variables that affect the extraction of urobilinogen. The results obtained with this method require less time, are reproducible and show a linear relationship to the direct Ehrlich's reaction values.

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ANNOUNCEMENT

The Mary Francis Gridley Scholarship and/or Loan Fund of the District of Columbia Society of Medical Technologists is pleased to announce the fourth $250 scholarship for a three month training program in specialized histological technics, to be given at the Armed Forces Institute of Pathology, Washington, D. C., beginning in January 1960.

The purpose of this scholarship is to help defray living expenses during the three month period, as no fees are charged for the course.

Applications may be submitted by any person with the following qualifications: 1. certified by the Board of Registry of the American Society of Clinical Pathologists as a medical technologist or an histopathologic technologist. 2. presently working in the field of histology.

Applications should be received by 8 December, 1959 and may be sent to:

Evelyn F. Ballou, Chairman
The Mary Francis Gridley Scholarship and/or Loan Fund
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SOME BASIC ASPECTS OF CLOT DISSOLUTION†

M. JUNE CALDWELL, M.T. (ASCP), R. C. HORN, JR., M.D.
SHIRLEY A. JOHNSON, Ph.D.
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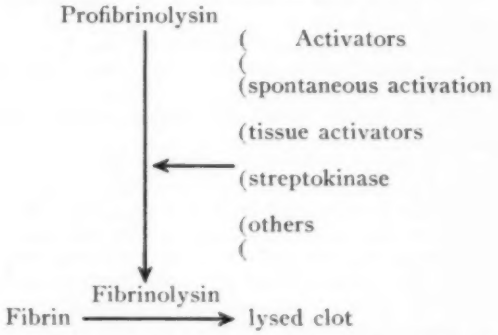
From a survey of the literature, it is apparent that intravascular clotting is being studied from two diverse approaches, from that of clot formation on the one hand, clot dissolution on the other. Both lines of investigation are very complex, but it appears now that more attention is being paid to the problem of clot dissolution. For this reason an understanding of the problems and the ability to use some of the simple fibrinolytic tests is becoming important to the laboratory technologist.

A simple scheme of clot dissolution is sketched below.



Both profibrinolysin (plasminogen) and fibrinolysin (plasmin) are present in plasma, profibrinolysin being in the greater amount (Astrup, 1956). Profibrinolysin is the precursor for the active enzyme fibrinolysin. The origin and nature of the activator in plasma is not known, although many substances are known which will activate profibrinolysin to fibrinolysin. Streptokinase is the best known activator but it is probably not the natural one. Urokinase, which has been found in urine, may be the natural activator but it has never been isolated from plasma. (Von Kaulla, 1958) Tissue activators have also been considered as the natural activator. (Astrup, 1956)

Since fibrinolysin is available for clinical trial in cases of intravascular clotting, some laboratory measurement of the level of activity of this enzyme would be helpful in following the infusion of this material. However, the laboratory measurement of fibrinolysin is complicated by the relationship of fibrinolysin activity to the fibrinogen values. For a clot may appear to dissolve when the fibrinogen level is too low to hold the red cells in a firm clot. (Krevans, 1957)

This paper is a brief discussion of fibrinolysin assays that can be used in the laboratory to give some indication of the active enzyme fibrinolysin which is being infused to dissolve an intravascular clot.

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† Second Award in Hematology, Scientific Products Foundation. Read before the 27th Annual Convention of ASMT, Phoenix, Arizona, June, 1959. Received for publication Feb. 28, 1959.

MATERIALS AND METHODS

Fibrinogen	Armour bovine fibrinogen prepared in a 1% solution of 10% imidazole buffer in saline.
Thrombin	Parke, Davis and Co. topical thrombin diluted to contain 20 units of activity per ml.
Fibrinolysin	Active enzyme, Actase, Ortho Pharmaceutical Corporation.
BAME	Benzoyl L-Arginine Methyl Ester.

The BAME esterase assay procedure for measuring fibrinolytic and profibrinolytic activity is very good for determining the activity of purified or partially purified substances. The presence of inhibitors does not interfere with this assay. (Sherry, 1957) This method, however, is not satisfactory for measuring activity in plasma; perhaps this is due to the varying lipid content of plasma which alters the turbidity accordingly.

A gross determination of fibrinolytic activity can be made by observing a clotted blood specimen during a 24 hour period at 37° C. There are many variables in this method of measurement. The fibrinogen level and erythrocyte numbers both influence the strength of the clot. Unless there is strong activity and lysis is complete, the determination is influenced by the judgment of the individual technician.

The method which involves adding fibrinogen to plasma and clotting with thrombin and then determining the time necessary for lysis to begin eliminates some of the pitfalls of whole blood procedures. This procedure is performed as follows: (Singher, 1959)

1. Place in small clotting tubes 0.4 ml. plasma
2. Add 0.2 ml. 1% fibrinogen
3. Blow in thrombin, start stopwatch, and shake to mix
4. Invert every 30 seconds to look for lysis
5. End point is reached when the clot falls away from the tube as it is inverted.

The time required for lysis to first appear is compared with that of an active enzyme substrate. Saline dilutions of Actase, which contain known units of activity, are used in the test procedure instead of plasma. One of the major disadvantages of this method is that the addition of fibrinogen necessitates a high degree of enzyme activity to show lysis and small amounts of active enzyme may easily be missed.

Methods for plasma clotting time, prothrombin time, plasma clotting time and fibrinogen determination were performed according to methods discussed by Caldwell (1957).

Results

A blood specimen sent to the laboratory for a routine prothrombin time showed a result of 29 seconds with the control 15 seconds, but the clot did not appear normal. The patient was not on anticoagulant therapy. Further investigation revealed a Lee White whole blood clotting time of 17 minutes compared with a normal of 10 minutes; a plasma

clotting time of 145 seconds was within the normal range of 90 to 150 seconds; the prothrombin consumption time was 13 seconds (normal is over 30 seconds and abnormal less than 20 seconds in this laboratory); the fibrinogen value was only 14 mgs. per cent. The clot formed in whole blood lysed within an hour in a 37° C. waterbath. Fibrinolytic activity of the plasma, determined as described above, was demonstrated in 29 minutes. This corresponded to approximately 40 units of activity of the Actase standard.

A second patient with a plasma clotting time of 150 seconds, a one-stage prothrombin time of 19 seconds, and a normal prothrombin consumption had a peculiar bleeding problem. On re-study two years later the prothrombin time was 23 seconds and the plasma clotting time 210 seconds. At this time the patient had a very active fibrinolytic enzyme.

DISCUSSION

Plasma inhibitors interfere in any test available for routine determination of fibrinolysis. The fibrinolytic activity is in the globulin fraction of plasma while the inhibitors are present in albumin fraction. It is reported that a euglobulin preparation of plasma does not contain these inhibitors of whole plasma (Siegel, 1957), so that a test on the euglobulin fraction might show more fibrinolytic activity.

The method of determining the fibrinogen level involves converting fibrinogen to fibrin and calculating the fibrinogen level from the tyrosine content of the fibrin. The fibrinogen level of 14 mgs. per cent in the first patient is probably low because the fibrinolytic enzyme was lysing the fibrin before it could be measured.

The results found in the second patient above seem to indicate that the time at which a blood sample is examined will give varying results. Similar data and ideas have been expressed by other investigators. (Phillips, 1958)

The usual subjective endpoint for these tests is a decided disadvantage. What is needed is a simple colorimetric test that can be done on any laboratory colorimeter, such as the Klett or Coleman.

When a fibrinolytic test is being used to follow intravenous infusion of the active enzyme, fibrinolysin, it is necessary to take great care with the blood sample. The active enzyme, as such, is very labile and the assay must be carried out as soon as the blood is drawn or the plasma must be frozen immediately.

SUMMARY

Importance of fibrinolysin testing in the laboratory is discussed. Possible methods are described and illustrated. The interference of fibrinolytic activity in the determination of fibrinogen is emphasized.

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A MICRO-MODIFICATION OF THE ANTHRONE TEST FOR SERUM SAMPLES OF LIMITED QUANTITY*

C. D. STIENECKER, B. Sc., and M. S. RHEINS, Ph.D.
Dept. of Bacteriology, Ohio State University, Columbus, Ohio

INTRODUCTION

In the course of experimentation with tuberculous rabbits in this laboratory, it became necessary to determine the total serum saccharide levels of the test animals at weekly intervals with reference to relative fluctuations. Classical macromethods for blood sugar determinations were impractical since only small quantities of serum were available for this procedure (the same serum samples were employed for other determinations). Therefore a precise micromethod for quantitatively determining serum saccharides and requiring but minute quantities of serum, ideally less than 0.5 ml., appeared desirable. For this purpose, a simple procedure utilizing the standard anthrone test reagent was developed which requires only 0.1 ml of serum and exhibits the accuracy and sensitivity of more established macromethods.

MATERIALS AND METHODS

Anthrone Reagent—This reagent is prepared by adding 2 grams of anthrone to one liter of 95% sulfuric acid. It was found that the reagent was stable at refrigerator temperature for a period of not more than two days and the aliquot of this stock needed for the test was therefore warmed to room temperature just prior to use.

Sera—Quantities of normal rabbit sera were obtained through the cooperation of the personnel in the Department of Bacteriology of The Ohio State University. Volumes of normal pooled human sera (600-800 individuals) as well as a number of individual samples were procured through the cooperation of the Ohio State Department of Health. With the Anthrone Method described by Morris[2], normal rabbit serum diluted 1:50 in distilled water (as the source of saccharide) resulted in satisfactory color production. Thus 0.1 ml of the test serum in 4.9 ml of distilled water satisfied the desired serum requirement range and in so doing negated the adverse optical effects of serum turbidity which varied with samples. The amounts of reagents employed were then diminished, retaining the 2:1 ratio, until it was established that 1.0 ml of the diluted serum sample and 2.0 ml of the anthrone reagent elicited adequate color production.

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method with regard to demonstrating, precisely, minute quantities of saccharide. Secondly, a standard for establishing the reproducibility and validity of replicate determinations was necessary in order to calculate the operative per cent error range. Finally, since it is usual with blood sugar determinations to express the results as a relative amount of a single saccharide, a conversion scale was desirable. To obtain a graph showing transmission percentages, 0.0-100 gammas, a titration of the glucose standard was performed in 13 mm x 100 mm test tubes containing 0, 5, 10, 20, 30, etc. gammas or 0, 0.05, 0.1, 0.2, 0.3, etc. milliliters of the standard respectively. To each tube was added sufficient distilled water to bring the volume to 1.0 ml, after which 2.0 ml of the anthrone reagent was added with thorough mixing. After standing 10 minutes, the mixtures were transferred to Coleman cuvettes, and read in a Coleman Junior Spectrophotometer Colorimeter at 620 millimicrons, previously set at 100% transmission with tube number one (blank). The per cent transmission of each dilution was recorded and graphed on semi-log paper. See Fig. I and Table I.) Replicate determinations established that the range of reproducibility varied no more than 2% transmission, the curves paralleling that in Fig. I.

Anthrone Test Procedure—The test procedure was essentially that described for the standard curve determination, except that 1.0 ml of a 1:50 dilution of the unknown serum was substituted for the glucose

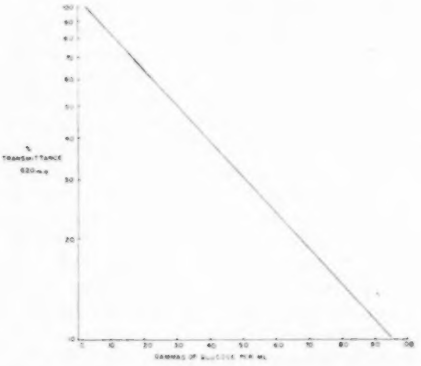

Fig. 1. Graphed values for standard glucose curve.

TABLE I

Values for standard glucose curve.

Tube	1	2	3	4	5	6	7	8	9	10	11
Gammas of Glucose/ml.	0	5	10	20	30	40	50	60	70	80	100
Percent Light Transmission	100	92	80	63	50	39	31	24	19	15	7

dilutions. A distilled water blank and a glucose standard, 50 γg./ml were included with each set of test samples. The tests were read and the per cent transmission converted to mg% by the equation: glucose in mg% equals the reading in gammas times a factor of five (5).

Evaluation of the Method—The test method was compared with the standard Folin-Wu blood sugar test[1]. Protein-free filtrates were obtained from representative rabbit and human serum samples, which had been previously treated and stored under various conditions, and the saccharide content of each was determined by both the anthrone and Folin-Wu methods. The results were compared to determine the quantitative limitations of the anthrone test. Saccharide stability was measured after storage of test samples at room temperature, after freezing, and after heat inactivation at 60C for 3 minutes.

RESULTS AND DISCUSSION

The results recorded in Table II were obtained by the two methods on Folin-Wu filtrates of the indicated serum samples with the exception of those listed under, "Total Serum Saccharide Levels." These were the results of performing the anthrone test on diluted whole sera. It is evident that the validity of the anthrone method is largely dependent on the use of fresh serum, since it appears that freezing for as short a time as several days, as was done with the human sera, samples 1 and 2, lowers the detectable saccharide content. This effect on the Folin-Wu

TABLE II

Comparison of saccharide values obtained by Anthrone and Folin-Wu Methods.

Serum Source	Treatment	Sample No.	VALUES IN MG. PERCENT	
			Anthrone Method (Protein-Free Filtrate)	Folin-Wu Method
Human.....	Fresh	1	120	114
		2	110	108
Human.....	Heat inact. 60C 3 min.	1	115	117
		2	105	107
Human.....	Room temp. for 2 days	1	105	103
		2	20	16
Human.....	Frozen at —20C. 5 days	1	105	108
		2	100	107
Pooled Rabbit	Frozen at —20C. 6 wks.	3	11	16
		4	60	60
		5	40	50
		6	70	60
Rabbit	Fresh	1	170	160
		2	139	130
		3	150	145
Pooled Rabbit	Frozen at —20C. 8 wks.	1, 2, 3	70	....
TOTAL SERUM SACCHARIDES				
Human	Fresh	1	250	....
		2	210	....
Rabbit	Fresh	1	275	....
		2	240	....
		3	250	....

method was not pronounced although decreased in values were observed for both methods with prolongation of sample storage either by freezing or holding it at room temperature for several days. Conversely, heat inactivation of the test samples did not adversely affect the saccharide values obtained. The values found for the fresh normal rabbit sera were usually high although these results compared favorably with those obtained with the Folin-Wu procedure.

With regard to the data presented, it is felt that the anthrone test measures saccharide levels in protein-free filtrates with sufficient accuracy, as verified by the standard Folin-Wu method, so as to permit the assumption that it also measures total whole serum saccharides with a similar degree of accuracy. This being the case, we feel that the anthrone micro-method has practical value where total serum saccharides are to be determined and under circumstances where limited quantities of serum are available.

NOTES ON TECHNIQUE

There are several points in the procedure which should be given particular attention to assure the greatest degree of accuracy possible. The anthrone reagent is prone to light and oxidative decomposition and should be examined for resultant darkening of the solution before use. When sizable amounts of this reagent are to be used, it is suggested that it be prepared just prior to use, and, in any event, never stored more than two days. Similarly, the glucose standard should be examined for evidence of bacterial contamination and evaporation. Refrigeration, as with the anthrone reagent served as an added precaution in this respect. The precision afforded both the standard curve and the test determination is dependent on precise dilution technique. Serial dilutions are to be avoided as pipette "carryover" can produce error significant in the test range. We found that Kahn pipettes are excellent for making serum dilutions, delivering 0.1 ml to 4.0 ml of diluent. Finally, cleanliness of glassware is of utmost importance. All test tubes and pipettes were cleaned in sulfuric acid (dilute) for several hours, followed by thorough rinsing with distilled water.

SUMMARY

1. A micromethod utilizing Anthrone has been described for the quantitative determination of total serum saccharides in limited quantities of serum.
2. Data is presented establishing the accuracy of the Anthrone test method with sera stored under various conditions and with samples subjected to heat inactivation. The standard Folin-Wu blood sugar determination method served as the comparison test.

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ROUTINE CLINICAL LABORATORY USE OF AN IN VITRO RADIOISOTOPE PROCEDURE†

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The *in vitro* erythrocyte uptake of I-131 labeled 1-triiodothyronine was first described by Hamolsky, Stein and Freedberg.[8] Crispell, et al., have also reported their results with this procedure.[3,4] The purpose of this report is to introduce to the medical technologist, interested in the use of radioisotopes as an aid in diagnosis, a basically new isotope technical procedure, modified for use in a routine laboratory. It differs from other chemical laboratory procedures only in that one must acquire the technique of handling radioactive material.

The results given in this paper were obtained over the past fifteen months in the routine laboratory of a small clinic equipped for isotope studies. The test was chosen because it 1) measures another parameter of thyroid function, 2) is not materially affected by recent administration of iodine containing compounds to the patient, and 3) holds promise of being an inexpensive screening test for thyroid function, eliminating the administration of radioisotope to the patient.

The test was originally performed[8] by adding the labelled hormone in duplicate to 3 ml. of blood in erlenmeyer flasks, which were then rotated for two hours in a 37° C. water bath. From each flash 1.0 ml. was taken in duplicate and the radioactivity measured before and after washing the erthrocytes in saline. To make the procedure applicable to a routine laboratory, we have modified it as follows:

MATERIALS AND METHODS

1) 5.0 ml. of blood is drawn (fasting not necessary) into mixed oxalate.* The test is performed within 48 hours to avoid significant lysis of the erythrocytes. 1.0 ml. of whole blood is pipetted into tubes in duplicate.
2) 0.05 ml. of a stock solution of chromatographically pure I-131 labelled 1-triiodothyronine dissolved in propylene glycol** is withdrawn with a tuberculin syringe and diluted with sufficient normal saline to give a specific activity of 3×10^4 c.p.m./0.1 ml.
This is done by the following formula:

uc/ml. $\times$ 0.133 $\times$ Decay factor = ml. saline stock solution

A sample calculation is as follows:

(Assay value of stock solution—470 uc/ml.; Scintillation well counter standardized to give 8×10^5 c.p.m./uc.)

1 ml. = 470 uc.

0.05 ml. = $470 \times 0.05 \times 8 \times 10^5$ c.p.m.

0.05 ml. = 18.8×10^6 c.p.m.

Since 3×10^4 c.p.m./0.1 ml. of the resulting solution is the desired strength, the number of ml. of saline to be added would be:

$$\frac{18.8 \times 10^6 \times 0.1}{3 \times 10^4} = 62.7 \text{ ml.} \times \text{physical decay since time of assay.}$$

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* Mixed oxalate: Ammonium oxalate 2.4 gm., potassium oxalate 6.0 gm/200 ml. water.
** Obtain from Abbott Laboratories: Oak Ridge, Tennessee.
† Read before the 27th Annual Convention of ASMT, Phoenix, Arizona, June, 1959. Received for publication January 30, 1959.

By calculation, we have reduced the variables to arrive at the constant of 0.133. For practical purposes, a calculation is made on the first day a shipment is used and only the decay correction applied thereafter, much simplifying the daily calculations.

3) 0.1 ml. of the diluted hormone solution is added to each tube.
4) The tubes are then shaken in a rack rotator in a 37° C. water bath for a period of two hours. This apparatus was designed in our laboratory—it consists of an Eberback variable rotator to which a metal bar is attached, suspending a rubber rack in the water bath.
5) Using a scintillation well counter, the total radioactivity in each tube is measured*.
6) After centrifugation, the supernatant plasma is removed, and the erythrocytes washed five times with ten-fold volumes of isotonic saline.
7) The radioactivity remaining in the erythrocyte fraction is then similarly counted.
8) The percent "erythrocyte uptake" is then calculated as follows:

$$\frac{\text{Net counts in washed erythrocytes} \times \text{100 hematocrit}}{\text{Net counts in whole blood} \times \text{patient's hematocrit}} \times 100 = \%$$

For purposes of comparison, the uptake is corrected to a reading of 100 hematocrit. The two determinations are averaged. If the difference between duplicates is greater than 3% uptake, the results are not reported.

Tests were performed on personnel, patients who were scheduled for thyroid studies, (I-131 thyroid uptakes, serum protein-bound iodine, basal metabolism) and other routine patients. Blood samples were received from the isotope department of a local hospital and from a local obstetrician, (cases of doubtful pregnancy, threatened and suspected abortion, and menstrual disorders). Thyroid status on nearly all patients was determined independent of the *in vitro* studies.

RESULTS

383 determinations were made on 319 adult subjects (seventeen subjects whose clinical status could not be determined were excluded). The effect on the results of the thyroid functional status of the patient is shown in Table I.

Euthyroidism: The euthyroid range is significantly higher in males than in females. Therefore, the mean of the male or female group, as indicated, as well as the over-all mean for the euthyroid group were used in further calculations. One euthyroid man was given thyroid stimulating hormone prior to his erythrocyte uptake, which was 14.8%.

Thyrotoxicosis: The total series of twelve observations is small but the average value is significantly different from the euthyroid range. With improvement in the toxic state, one patient's uptake decreased from 24.3 to 18.7% and another from 19.7 to 17.7%. Although only three toxic females were tested their results (mean 16.3%) were significantly

* The samples are counted for a sufficient period of time to obtain a counting error of less than 1%, with correction for background radiation.

TABLE I

The effect of thyroid status on the *in vitro* erythrocyte uptake of I-131 labelled 1-triiodothyronine.

Thyroid Status	No. Pts.	No. Tests	Range Percent	Average ± S.D. (Percent)
Euthyroid (total)	272	272	10.1—23.0	15.4 ± 2.24
Male	125	125	11.9—23.1	16.4 ± 2.24
Female	147	147	10.1—19.1	14.6 ± 1.93
Thyrotoxicosis	10	12	17.1—24.4	20.5*
Hypothyroidism	46	47	9.3—16.2	12.3 ± 1.38
Euthyroid with recent iodine administration	11	20	13.5—22.0	16.2 ± 2.10

* The small series did not justify statistical analysis of variance.

lower than the results on the nine males (mean 21.9%). More cases of thyrotoxicosis need to be examined before more definite conclusions can be drawn.

Hypothyroidism: The average uptake is significantly lower than the euthyroid range with a small overlap. There is no difference between males and females. One subject was frequently tested and it is of interest that she developed clinical hypothyroidism while under observation and responded to thyroid medication. Her erythrocyte uptakes are shown in Table II; they also demonstrate the effect of menstruation.

Previous Iodine Administration: In three of the eleven subjects, the thyroid uptake of I-131 done simultaneously, demonstrated the expected depression. The mean uptake following iodine administration is significantly higher than the euthyroid range, but not sufficiently so to prevent the test from being useful, especially since the other thyroid tests (serum protein-bound iodine and I-131 thyroid uptake) are so markedly affected.

TABLE II

Results on a patient who developed hypothyroidism while under observation. The effect of hypothyroidism, therapy with thyroid substance, and menstruation are demonstrated.

	Erythrocyte Uptake (Percent)	Clinical Status
10/21/57	12.6*	Euthyroid
11/ 1/57	15.3	Euthyroid
11/ 6/57	15.1	Euthyroid
11/18/57	12.1*	Euthyroid
12/16/57	12.0*	Euthyroid
1/10/58	13.1	?
4/29/58	10.2*	?
7/16/58	12.5	Hypothyroid
10/ 8/58	10.8	Hypothyroid (Thyroid uptake of I-131 6 hrs.—4.4%; 24 hrs.—10.9%)
1/ 7/59	15.4	Euthyroid (Thyroid 2 grains daily)

* Menstruating.

Hashimotos: Two patients were examined. Their results were 11.4% and 11.4%. One of these patients was hypothyroid, the other euthyroid by other tests.

Non Toxic Nodular Goiter: Five patients (two males and three females) were studied. The results were 13.8, 13.9, 14.5, 15.7 and 16.8%, averaging 14.9%. All patients were euthyroid by other procedures. They do not differ significantly from the euthyroid range.

Acromegaly: One man, euthyroid by other observations, had an erthrocyte uptake of 17.4%.

Hypo-Pituitarism (chromophobe adenoma): One man, hypothyroid by all other observations, had an erythrocyte uptake of 10.4%.

Estrogen Administration: Estrogen was given to three women before the erythrocyte uptake: the results were 13.2, 14.4 and 9.1%, averaging 12.2%.

Menstruation: It was observed, early in the experiment, that the erythrocyte uptake is low during the menstrual period. Data on five patients is given in Table III. In all, thirteen women had eighteen determinations during menstruation. The results ranged from 8.5 to 15.0%, averaging 11.9% ± 1.34.

TABLE III

The effect of menstruation and pregnancy on the erythrocyte uptake.

A	B	C	D	E	F	G
15.5.....	13.1*	14.3	15.3	13.0†	8.0 (4 mo. gest.)	12.0 (8 mo. gest.)
14.8.....	15.4	14.2	12.6*	8.7*	7.9 (5 mo. gest.)	9.3 (9 mo. gest.)
13.3*....	11.2*	11.9*	15.1	....	6.8 (6 mo. gest.)	.... Delivered at term 2 weeks later.
	10.0 (2 mo. gest.)	....	12.1*	....	9.4 (8 mo. gest.) Delivered at term.	17.5
	7.8 (5 mo. gest.)	....	12.0*			
	Delivered at term.	....	....	....	13.2 13.9	....

* Menstruating.
† Began menstruating 12 hours later.

Amenorrhea: One patient with secondary amenorrhea (not pregnant) had an erythrocyte uptake of 10.7%.

Pregnancy: During pregnancy there was a great decrease in the erythrocyte uptake. Six pregnant subjects were studied, three of these are listed in Table III. Subjects F and G were followed during and after pregnancy. The twelve determinations during pregnancy ranged from 6.8 to 12%, averaging 9.3%. One subject aborted the day following our study. One patient, known to be a habitual aborter had an uptake of 13.0% at 4 weeks gestation. She is still pregnant 4 months later, but continues to threaten to abort. The time at which the erythrocyte uptake becomes depressed during pregnancy is not known. Two subjects were studied at 4 weeks gestation, the results were 11.9 and 13.8%. By

two months the uptake seems to be uniformly depressed. In fact, this test may be helpful in diagnosing early pregnancy.

Liver Disease: Two patients were studied during hepatitis: one man had results of 13.7, 16.0%; a woman's uptake was 11.4%. In one woman dying of liver failure from metastatic breast cancer, the uptake was 10.4%. Another woman, with chronic hepatitis had an uptake of 9.5%. The average value of the five determinations in the presence of liver disease was 12.2%.

Tests of significance were carried out to determine whether or not a significant difference existed among the various groups. One can test the significance of the differences between the means by calculating "t".*

$$*\text{"t"} = \frac{[\overline{X}_1 - \overline{X}_2] \cdot \sqrt{\frac{N_1 \cdot N_2 (N_1 + N_2 - 2)}{N_1 + N_2}}}{\sqrt{S(X_1^2) - \frac{[S(X_1)]^2}{N_1} + S(X_2^2) - \frac{[S(X_2)]^2}{N_2}}}$$

The evaluation is given in Table IV. This was done because of the wide variation in numbers of observations.[2]

In all groups, there was good correlation between erythrocyte uptake

TABLE IV

Levels of Significance (t) between means of various groups

Groups To Be Compared	Euthyroid Female	Euthyroid Male	Euthyroid Total	Hypothyroid Female	Hypothyroid Total
Euthyroid famales	....	16.2*h*	....	....	....
Euthyroid males	16.2*h*	....	....	....	....
Toxic males	....	8.1*h*	17.5*h*	....	....
Toxic Total	....	....	14.8*h*	....	....
Hypo female	13.0*h*	....	11.3*h*	....	....
Hypo male	....	6.5*h*	9.4*h*	....	....
Hypo Total	....	....	18.2*h*	....	....
Iodine female	3.0†	....	1.2	....	....
Iodine male	....	0.97	3.8‡	....	....
Iodine Total	....	....	10.0*h*	....	....
Thyroid female	7.0*h*	....	6.6*h*	1.7	....
Thyroid Total	....	.....	7.3*h*	....	1.7
Liver disease Total	....	....	7.1*h*	....	0.12
NTNG Total	....	....	1.2	....	3.5†
Menses	9.5*h*	....	14.4*h*	0.66	....
Pregnancy	14.6*h*	....	19.2*h*	5.6*h*	....

* Significant at 5% level.
† Significant at 1% level.
‡ Significant at 0.1% level.
h Significant beyond the 0.1% level.

and serum protein-bound iodine, I-131 thyroid uptake, serum cholesterol and basal metabolic rate. Indeed, in some instances the erythrocyte uptake appeared to correspond more closely with the clinical impression than did the other tests.

DISCUSSION

In the performance of any isotope test, certain safety precautions must be observed in order to reduce the hazard of contaminating the laboratory and personnel.[1] The stock solution must be stored within a sufficiently heavy lead container, rubber gloves must be worn when handling the isotope solutions, and a remote pipetter* used for transferring the radioactive material. Furthermore, there should be a definite space in the laboratory for performance of test and all isotope equipment must be cleaned and stored separately. Medical technologists performing the test should routinely wear film badges and have blood counts, prior to exposure and every three months. The laboratory should periodically be monitored to ascertain the adequacy of shielding and disposal procedures. Any medical technologist can learn techniques easily, but by law, these must be done under supervision of a person licensed by atomic energy commission to obtain and use isotopes. Radioactive isotope procedures are applicable to a routine clinical laboratory.

The circulating thyroid hormone (primarily thyroxine and triiodothyronine) is bound to a plasma protein, between alpha-1 and alpha-2 globulin, electrophoretically (thyroxine-binding protein).[7, 11, 12] Hamolsky performed fifty-six criss-cross experiments, demonstrating that the erythrocyte uptake was determined by the thyroidal state of the patient from whom the plasma was obtained.[5] It appears that the plasma protein binding is the major factor with erythrocyte "taking up," in some manner the remaining hormone.[4] This is generally demonstrative of over-all thyroid function. The theoretical possibility of understanding the differences between diffuse toxic goiter (Grave's disease) and nodular toxic goiter (Adenoma) patients has been suggested.[9] The interesting findings in menstruation and pregnancy and their disorders may give rise to a better undertsanding of the part played by plasma proteins in these conditions.[5, 6, 10]

It is possible with the *in vitro* erythrocyte uptake, simply and rapidly to assess the thyroid function of most patients. It avoids administration of isotope to the patient, and is little influenced by administration of iodine in contrast to the protein-bound iodine and thyroid uptake of I-131. In some of our patients who were under treatment for either hypothyroidism or hyperthyroidism, the results of this test correlated better with the thyroid status than did other methods, changing with the clinical improvement of the patient during therapy. Thus, it appears that the *in vitro* erythrocyte test may be of diagnostic value in cases where certain factors complicate the interpretation of other thyroidal diagnostic procedures.

SUMMARY

1. It is possible and practical to perform a simple radioisotope procedure in a routine clinical laboratory.

* Propipettor.

2. The erythrocyte uptake of I-131 labeled 1-triiodothyronine procedure was modified for routine use and performed 383 times on 319 patients.
3. The results of the tests are given, statistically analyzed, and compared with other thyroid function tests.
4. It is an inexpensive thyroid function test which eliminates the administration of radioisotope to the patient. The validity of the test is not materially changed by administration of iodine containing compounds.
5. Interesting findings are noted during pregnancy and menstruation.
6. Further experience with the test is necessary to determine its full place in the diagnosis of thyroid diseases, but its applicability to a routine laboratory has been established.

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ADDENDUM

Since completion of this paper, an article by Hamolsky, et al., has been published* giving results of their observations over a four year period. This report describes variable results using blood with hematocrit values outside the range from 30-55 mm.; such blood specimens were, therefore, corrected to hematocrit values within this range by the

* Hamolsky, Milton W., Golodetz, Arnold and Freedberg, A. Stone: The plasma protein-thyroid hormone complex in man: III. Further studies on the use of the **in vitro** red blood cell uptake of 1-131 triiodothyronine as a diagnostic test of thyroid function. J. Clin. Endocrinol, Vol. 19, 103-116, Jan. 1959.

removal or addition of the same individual's plasma prior to addition of labelled 1-triiodothyronine.

Studies have been instigated in our laboratory to further clarify these observations and to determine at what hematocrit level, altered results occur in the *in vitro* uptake. This will be reported at a future date.

ACKNOWLEDGMENT

The author wishes to acknowledge gratefully the advice and helpful criticism of Dr. L. R. Robbins and is indebted to Dr. David Vinson for his assistance in preparing the statistical analyses.

A paper presented from the clinical standpoint currently appears in the October 1959 JOURNAL OF CLINICAL ENDOCRINOLOGY AND METABOLISM on page 1292. This paper, entitled, "Experience with In Vitro Erythrocyte Uptake of I^{131} Labeled 1-Triiodothyronine in a Routine Clinical Laboratory," by Leonard R. Robbins, M.D., naming Miss Murphy as technical assistant, was submitted for publication at a later date (March 18, 1959) than this one, so the tables will be based on more determinations.

IDEAS UNLIMITED

From time to time our readers have asked why we don't devote a page of THE AMERICAN JOURNAL OF MEDICAL TECHNOLOGY to "LETTERS TO THE EDITOR," "LABORATORY SUGGESTIONS," "HELPFUL HINTS" —or something of the kind.

Such a section—to be successful—depends on YOU—on YOUR Contributions.

Do you have the IDEAL ———?? Mrs. Margaret Phillips, Des Moines, Iowa, Chairman of the A.S.M.T. Standards and Studies Committee, says, "I have yet to find a satisfactory 'pick-up' tray." How about you? Send in a sketch—picture, diagram or write-up. The description must be brief —to occupy one page or less of the journal. When preparing your material, comply with publication requirements by following the directions on page II of every issue.

PHENYL-ETHYL-ALCOHOL MEDIUM AND ANTIBIOTIC SENSITIVITIES OF MIXED BACTERIAL FLORA**

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The illness accompanying bacterial invasion of the genito-urinary tract is most often acute. Whether the causative organisms are of hematogenous or urogenous origin, prompt, effective treatment is indicated, in order to prevent damage. The attending physician is reluctant to delay antibiotic therapy until he receives the results of sensitivity studies from the Bacteriology Laboratory. The urgency of this situation is well-founded. A drug may be selected on the basis of a gram-stained smear. However, due to the natural tendency of micro-organisms to develop resistance to commonly used antibiotics, the initial drug chosen may not be the "drug of choice."

In-vitro sensitivity tests are a valuable aid to the internist in his selection of one or more weapons with which to fight infection. It is, therefore, the grave responsibility of every bacteriologist to adopt reliable methods which will allow this information to reach the physician in the least possible time.

In most instances, the urine specimens from patients with acute or chronic cystitis, pyelonephritis, or urethritis, yield mixed cultures of gram-negative and gram-positive bacteria. Due to the more rapid growth of the enteric bacilli, the isolation of pure cultures for testing the antibiotic sensitivity of species of Streptococcus and Staphylococcus is time consuming.

A report that the gram-positive micro-organisms are present may be sent out after a few hours have elapsed, but the antibiotic sensitivity of these invaders often cannot be determined for at least 48 to 72 hours. If the susceptibility tests are set up on the nutrient blood plates required for the fastidious Streptococci, and gram-negative rods are also present, overgrowth of these bacilli will obscure the reading of the zones of inhibition of the less virile Streptococci. This same masking effect also occurs on an antibiotic sensitivity agar diffusion test where Staphylococci are plated in the company "spreader" types of Enterobacteriaceae.

Phenyl-ethyl-alcohol medium* is a nutrient substrate which will inhibit the growth of gram-negative organisms for 36 hours, or longer, but will permit excellent growth of Staphylococcus and Candida species. If phenyl-ethyl-alcohol medium is prepared with 5% defibrinated blood, it will support the growth of species of Streptococcus, Pneumococcus, and the Diphtheroid bacilli.

The problem was posed that this medium might be used to advantage in agar-diffusion sensitivity tests of cultures of mixed populations, thus enabling the Bacteriology Laboratory to report results to ward physicians in a maximum of 24 hours. Several urine cultures of mixed gram-negative and gram-positive micro-organisms were tested, in duplicate. Pnenyl-ethyl-alcohol blood plates and trypticase-soy-agar* blood plates were seeded with suspensions containing both types of bacteria, and antibiotic

* Baltimore Biological Laboratories.

** Second ASMT Award. Read before the 27th Annual Convention of ASMT, Phoenix, Arizona, June, 1959. Received for publication Feb. 28, 1959.

disks placed on each plate. Tests were incubated overnight at 37° C.

Results were gratifying. The gram-negative rods were well inhibited on the phenyl-ethyl-alcohol plates and clear-cut readings of zones of inhibition of the gram-positive cocci were possible. Responses of the enteric bacilli were noted from the zones around the antibiotic disks on the trypticase-soy-agar plates.

It is well known that some chemical compounds exert an antagonistic effect upon the action of many antibiotics. Traces of some chemicals in artificial media will alter the enzyme systems of bacteria and change their responses to biochemical tests. (Davis & McDermott—1952) Phenyl-ethyl-alcohol is an aromatic alcohol, synthesized by the interaction of benzene and ethylene-oxide, by means of the *Friedel-Crafts* formula (Lowy and Harrow—1951). The presence of a primary alcohol radical ($CH_2 \cdot CH_2 OH$) in this compound presupposes of the possibility of (1) an adverse influence on the antibiotics tested, and (2) impediment of growth of test organisms by inhibition of inherent functions essential to life. Before the establishment of this method for clinical use, a comparative study was made to ascertain whether any differences could be noted in the susceptibility of gram-positive bacteria on medium containing phenyl-ethyl-alcohol and on medium free of the alcohol.

MATERIALS AND METHODS

Antibiotics

Ten of the therapeutic agents most commonly administered clinically were selected for this study. Bacto-Sensitivity disks*, containing three concentrations of each of the antibiotics were used.

These are listed below.

Achromycin—10, 30, and 60 mcg, Bacitracin—2, 10, and 20 units, Chloromycetin—10, 30, and 60 mcg, Dihydrostreptomycin—1, 10, and 100 mcg, Furadantin 25, 50, and 100 mcg, Penicillin—0.5, 1, and 10 units, Polymyxin B—5, 10, and 30 mcg, Neomycin—5, 10, and 30 mcg, Terramycin—10, 30, and 60 mcg, and Erythromycin—10, 30, and 60 mcg.

The dilutions are considered as low (L), medium (M), and high (H) and are so designated in the following discussions.

Media

For stock cultures and test bacterial suspensions, 3% trypticase-soy-broth was dispensed in 5 ml. volumes in 13 mm x 120 mm screw-capped culture tubes.

For the agar plate susceptibility testing, three media were prepared:

(1) Trypticase-soy-blood agar base,

(2) Tryticase-soy-agar containing 5% citrated human blood,

(3) Phenyl-ethyl-alcohol medium, with 5% citrated human blood.

Note of caution: When preparing the phenyl-ethyl-alcohol basic medium, **care** must be taken in sterilization. Overheating, or prolonged heating, completely destroys the capacity of this substrate to inhibit gram-negative bacilli. 12 minutes at 110° C is optimum.

Plates, each containing approximately 95 ml. of medium were poured in 150 mm petri-dishes and incubated at 37° C for 18 hours to check sterility.

*Difco Laboratories.

Cultures

The bacteria used in these studies were isolated in pure culture from 47 patients with genito urinary tract infections. Following are the names and numbers of strains of the micro-organisms cultured: Proteus species, 8, Pseudomonas *aeruginosa*, 8, Aerobacter *aerogenes*, 4, Klebsiella *pneumoniae*, 4, Escherichia *coli*, 8, Staphylococcus *pyogenes*, var. *aureus*, 17, Staphylococcus *pyogenes*, var. *albus*, 3, Micrococcus species, 2, Streptococcus *pyogenes*, 9, Steptococcus *viridans*, 2, Streptococcus *faecalis*, 2, and Streptococcus species (non-hemolytic)—1.

Each strain was maintained as a stock culture in trypticase-soy-broth at 37° C, with frequent transfers.

Each gram-positive organism was paired with one of the gram-negative bacilli, and combinations made in broth suspensions, comparable to the mixed-populations that might result from the cultures of urines. 36 such cultures were prepared.

Preparation of test suspensions

For rejuvination of the stock strains, 0.1 ml. from each stock broth culture was transferred to a tube of trypticase-soy-broth, with a sterile pipette. After incubation for 18 hours at 37° C, the "mixed" and pure organisms suspensions were prepared. The 18-hour old broth culture of each strain of bacteria was serially diluted and 0.1 ml. of each added to tubes of trypticase-soy-broth in the following manner, for the sensitivity-test-suspensions:

Tube 1—one gram-positive organism, diluted 10^{-1}.

Tube 2—one gram-negative organism, diluted 10^{-3}.

Tube 3—the same pair of gram-positive and gram-negative organisms, of the same dilutions, which were added to tube 1 and tube 2.

Tubes were agitated gently to mix, and incubated for 4 hours at 37° C.

Each set of three tubes constituted an experimental series to be plated on the agar susceptibility test media. The higher dilution of the broth suspensions of the gram-negative bacilli was used to compensate for the more rapid growth curve of these organisms.

Preparation of the agar plate susceptibility tests.

Four plates of medium were utilized for each test. The 36 sets of three broth suspensions, four-hours old, were seeded as follows:

Plate 1—Trypticase-soy blood agar plate, from tube #1—pure culture of gram-positive coccus.

Plate 2—Trypticase-soy agar plate, no blood, from tube #2—pure culture of gram-negative bacillus.

Plate 3—Trypticase-soy agar blood plate, from tube #3—mixed culture.

Plate 4—Phenyl-ethyl-alcohol blood plate, from tube #3—mixed culture.

Using a sterile, cotton-plugged pipette, 0.5 ml. of the broth suspensions were pipetted on the center of the proper respective plates, and each streaked with a sterile cotton swab until the entire surface of the medium was swabbed. This amount of inoculum, and the manner of streaking, provides a relatively dry, but well seeded plate surface. Quadruplicate sets of antibiotic disks were placed on the plates of each

test with alcohol-flamed forceps. This procedure was carried out immediately after inoculation, in order to assure diffusion of drugs before bacterial growth could begin. A space of approximately 10 mm was allowed between each disk, and the set of three dilutions of each drug was positioned clockwise in the same order on every plate, in order to facilitate readings of tests. Readings were taken after an overnight incubation at 37° C.

Interpretation of susceptibility readings.

The presence of any sharp line of inhibited growth around an antibiotic disk was denoted as sensitive. No zone diameters were measured. An organism which grew in contact with a sensitivity disk was recorded as being resistant to that drug dilution.

RESULTS

Bacterial Growth

The amount of growth of the gram-positive cocci on the series of comparative plates was relatively constant. Zones of inhibition were as clear-cut and easily read on the phenyl-ethyl-alcohol plates as on the trypticase-soy-agar blood plates. The enteric bacilli were not completely inhibited on the phenyl-ethyl-alcohol plates of 3 tests but the growth was not sufficient to prevent accurate readings of the susceptibility of the Streptococci and Micrococci present. Only one phenyl-ethyl-alcohol plate, was overgrown by Pseudomonas. On this test it was not possible to take readings of the sensitivity of the Streptococcus. All the trypticase-soy-agar blood plates seeded with the mixed cultures were over-grown by the gram-negative rods, and the growth of the gram-positive cocci was obscured.

Action of antibiotics

The readings from the trypticase-soy-blood-agar plates denoting the susceptibility of the pure cultures of the gram-positive test bacteria were essentially the same as the readings from the phenyl-ethyl-alcohol blood plates indicating the reaction of the same organisms in mixed culture. The parallel sensitivity or resistance to the antibiotics on both media showed that phenyl-ethyl-alcohol did not exert any adverse effect on the reaction between the test bacteria and test drugs. Only five differences in zone readings occurred. On the phenyl-ethyl-alcohol blood plate, two strains of Staphylococcus pyogenes, var. albus, and three strains of Staphylococcus pyogenes, var. *aureus*, showed sensitivity to the lowest dilution of Bacitracin and Polymyxin. A resistant response to this dilution of these antibacterials occurred on the trypticase-soy-agar blood plate. The presence of the phenyl-ethyl-alcohol in the medium failed to influence the growth or susceptibility patterns of any of the strains of Streptococci. Identical readings were obtained from both the comparative media.

DISCUSSION

These comparative studies provided confirmation of the value of the use of a phenyl-ethyl-alcohol plate in conjunction with routine media when determining the in-vitro antibiotic sensitivity of the gram-positive

bacteria in biological materials infected with mixed populations of gram-positive and gram-negative micro-organisms. The failure to demonstrate any changes in susceptibility or resistance of the Streptococci and Micrococci to the most widely used antibiotics more than compensates for the five instances in which readings on the two types of test plates showed minor differences. The irregularities occurred in relation to the subclinical therapeutic dilution of drugs rarely used.

Obviously, the relatively uniform susceptibility readings of the gram-positive cocci from the phenyl-ethyl-alcohol plates, in contrast to complete obliteration of this information by the growth of the gram-negative bacilli on the inhibitory-free medium, indicate that phenyl-ethyl-alcohol medium is the medium of choice for in-vitro sensitivity tests of gram-positive organisms in cultures of mixed flora.

In this investigation, the amount of seeding was kept at a minimum, in order to keep all tests as quantitative as possible. In routine clinical procedures, however, a heavy inoculum may be used. This permits the tests to be read after 4 to 6 hours. Direct seeding from the centrifuged specimen of urine will permit the report of a susceptibility test of both gram-negative rods and gram-positive cocci to be made after an overnight incubation. The sensitivity of each micro-organism to therapeutic agents is of much more value to the physician than the identity of the organisms. The information as to genus and species can be sent out later.

SUMMARY

The suggestion that phenyl-ethyl-alcohol medium might prove useful as a routine clinical procedure for in-vitro susceptibility tests of gram-positive bacteria in cultures of mixed bacterial populations is presented. Experimental studies were made, using various strains of Enterobacteriaceae and gram-positive cocci in pure and mixed cultures. The methods used, the comparative media tested, and results of the evaluation are described. The effect of phenyl-ethyl-alcohol on the results of the sensitivity tests of the gram-positive micro-organisms compared to the same bacteria on alcohol-free medium is discussed.

These comparison studies indicate that the two media were found to be in agreement. No discrepancies between readings were observed, except in minor instances. These slight differences are mentioned.

It is proposed that a phenyl-ethyl-alcohol blood plate be used as an adjunctive medium when setting up the in-vitro sensitivity tests of gram-positive micro-organisms found in genito-urinary tract infections because:

(a) it provides an optimum condition of growth for gram-positive organisms, but suppresses the growth of gram-negative rods.

(b) the use of phenyl-ethyl-alcohol medium permits reports of the antibiotic sensitivity of both gram-negative and gram-positive organisms to go out in a maximum of twenty-four hours after specimens are received. We have used this as a routine clinical procedure in testing hundreds of mixed cultures for the past three years. Results have been highly satisfactory.

(c) phenyl-ethyl-alcohol exerts no antagonistic effect on the test antibiotics.

ACKNOWLEDGMENT

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* Veterans Administration Hospital, Louisville, Kentucky.

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FURTHER STUDIES ON THE ISOLATION AND IDENTIFICATION OF CANDIDA SPECIES*†

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The search continues for a simple, sure way to identify Candida species in a routine diagnostic microbiological laboratory. Laboratories vary as to methods and media used for the isolation and identification of these organisms. The fact that the majority of the eight to ten yeast cultures received by us each month from other laboratories for identification are *Candida albicans* indicates to us that some technologists are still having trouble identifying this organism.

Since 1955[2] our laboratory has used fermentation studies for identification of less than 5% of the isolated strains of *Candida albicans*. Since the rice extract, ABY, and Bi GGY agars[1] have become available, less than 1% of our strains have required fermentation studies for identification.

The data presented here were recently acquired as the result of a study done to re-affirm our technics used in the diagnostic mycology laboratory. We believe that they will help some of those technologists having trouble in identifying Candida species.

MATERIALS AND METHODS

The media used included Levine's E.M.B.,[6] dehydrated zein and rice extract agars,[1] zein agar made by us using the dehydrated zein,[4] and the "homemade" rice agar.[5] Also used were the commercial ABY and Bi GGY agars. Fermentation studies were done according to the method of Martin[3] as well as by means of the Key rapid fermentation tablets.

The E.M.B. plates were inoculated with pure cultures of species of Candida growing on Sabouraud's glucose agar slants by the streak dilution method and incubated in a candle jar at 37° C. The growth on these plates was examined at 24 and 48 hour intervals under 100X magnification for the presence of radiating mycelial fringe.

The rice and zein media were inoculated by using a bacteriological probe containing a *small* amount of inoculum, making two parallel cuts in the medium; care was taken to avoid picking up any of the inoculum from the preceding cut. The surface of the agar and the cuts were then streaked with the same probe. This technic is one that serves as an excellent dilution method resulting in only a few yeast cells being present in the last streak. A sterile coverslip was then placed over one end of the inoculated site. Each fungus was inoculated in duplicate. The plates were incubated at room temperature and read at 24, 48, and 72 hours for chlamydospore production.

The basal medium for the fermentation studies was beef extract broth containing brom-cresol purple as an indicator. Sugars used were 1% glucose, sucrose, maltose, and lactose. These carbohydrate media were inoculated by adding 0.1ml of a saline suspension of the organism from a second generation growth on beef extract agar slant followed by overlaying with melted sterile vaseline. These were incubated at 37° C for

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10 days and then examined for acid and gas formation.

A tube of Sabouraud's broth was inoculated along with the carbohydrate media, incubated at 37° C for 48 hours, at which time the type of growth was noted and the tube discarded.

Rapid Key sugars were used according to the manufacturer's instructions, inoculated in the same manner as was done for the regular carbohydrate broths, incubated at 37° C for 48 hours, read for acid and gas and discarded.

Attempts to use the ABY and Bi GGY media in the study of pure cultures of Candida species were unsatisfactory. Since the manufacturers advocate these media for the identification of *Candida albicans* and *C. tropicalis* growing on them from the original inoculum we decided to discontinue their use in the study of pure cultures. Thus all sputa received for mycological studies were inoculated directly to these media as well as to other indicated media. All Candida species appearing on these media had their identity confirmed by use of chlamydospore and fermentation media.

RESULTS

A total 100 freshly isolated strains of Candida species were included in this study: 66 were *C. albicans*, 5 were *C. stellatoidea*, 18 were *C. tropicalis*, 10 were *C. parakrusei*, and 1 was *C. krusei*.

On the E.M.B. medium 49% of the *C. albicans* and 55% of the *C. tropicalis* isolates were positive for "feathering." This confirms Haley and Stonerod's[2] earlier observations and explains why we do not use this medium in our laboratory. We are also concerned about the number of strains of *C. albicans* that do not develop the "feathering" on this medium.

Chlamydospore production by *C. albicans* was at its maximum—100%—when the commercial rice extract agar was used; the minimum production of these spores (79%) was seen when the commercial zein agar was used. The "homemade" rice extract enabled 97% of the *Candida albicans* strains to develop chlamydospores while 94% produced these spores when the "homemade" zein medium was used.

We were unable to obtain consistent, reproducible results when the Key fermentation tablets were used. We attempted to improve the method by overlaying the medium with vaseline but without success.

Candida albicans and *C. tropicalis* on ABY medium produces black colonies that have a beautiful metallic sheen; there is diffusion of the black pigment into the medium. On Bi GGY medium, *C. albicans* produces a large, flat, brownish-black colony without a metallic sheen; *C. tropicalis* has the same appearance as noted on ABY medium.

To date we have identified approximately thirty isolates of *C. tropicalis* and *C. albicans* on the Bi GGY and ABY media. Only one strain of *C. albicans* failed to yield the characteristic appearance on these media.

All other species of Candida are brown when seen on these media and require fermentation studies for their identification. The authors do not believe that there will be any trouble differentiating species of Cryptococcus and Torulopsis from Candida when these media are used.

DISCUSSION

On the basis of the data acquired from this and previous studies we have very little difficulty in identifying Candida species; indeed, less than 1% of our strains of *Candida albicans* require fermentation studies for

identification.

We would like to re-emphasize a point made previously[2] which will greatly effect the production of chlamydospores on any medium—it is essential that a small amount of inoculum be used and that the method of inoculation be one of continuing dilution of the inoculum. When a heavy inoculum is used the Candida species may fail to produce any pseudohyphae, much less chlamydospores; in such cases the organism is usually reported out as "not a Candida" and discarded. Our work with other filamentous yeasts has confirmed this observation. This failure to be concerned about the amount of inoculum used is without a doubt one of the reasons that some technologists are having difficulty identifying their strains of *Candida albicans*.

Another difficulty arises when one goes directly from a medium containing a reducing sugar such as glucose to a chlamydospore medium. When this is done there is the possibility that enough of the sugar is carried over by the fungus to block chlamydospore formation. This may be avoided by culturing the yeast on beef extract agar for 24 hours followed by a second sub-culture. Following 48 hours of growth on beef extract agar the fungus may then be inoculated on chlamydospore medium.

Most of the media on the market for use in the isolation and identification of Candida species are satisfactory; some are better than others, but it is impractical for any laboratory to use all of them. We have found the methods and media discussed in this paper highly satisfactory.

SUMMARY

1. In our laboratory E.M.B. has not proved to be a satisfactory medium for the identification of *Candida albicans;* a large percentage of these strains do not develop the "feathering" and many isolates of *C. tropicalis* will also develop the characteristic "feathering" on this medium.

2. The method of inoculating chlamydospore agar described in this paper definitely enhances the production of chlamydospores.

3. Rapid sugars have not been satisfactory in our laboratory; we believe the method of Martin is still the best method for the study of fermentation reactions of these yeasts.

4. In our laboratory the commercial rice extract agar appears to be the most satisfactory of the various extract agars for the production of chlamydospores by *Candida albicans*.

5. We are finding the ABY and Bi GGY media of Difco to be very satisfactory in the isolation and identification of *Candida albicans* and *C. tropicalis*. We do not recommend that they replace the chlamydospore medium but rather that they be used in conjunction with the latter medium.

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ADDENDUM TO THE NUMERICAL DESIGNATION OF STAPHYLOCOCCAL ANTIBIOGRAMS*

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A numerical designation of staphylococcal antibiograms has previously been proposed in which antibiotics are assigned a number in the mnemonic SPECT.[2] Resistance is denoted by use of the number, susceptibility by its omission. The purpose of this paper is to report an extension of this scheme to cover situations in which susceptibility results for all five of the selected antibiotics may not be available.

In reviewing and tabulating data of the past several years from the Dermatology Research Laboratory of this Department, it was found that many staphylococci had not been tested against all five of the antibiotics in the mnemonic SPECT, especially streptomycin (or dihydro-streptomycin) and chloramphenicol. The original scheme has proposed the use of suffixes for additional antibiotics. According to the Roman numerical system, letters suffixed to a basic number add to it, those prefixed subtract from it. Therefore, any omitted antibiotic susceptibility test could likewise be designated by appropriate prefixes. Thus, the designation SC25 indicates an organism resistance to penicillin and tetracycline, susceptible to erythromycin, but not tested with streptomycin or chloramphenicol.

The practice in this laboratory has been to record all organisms not susceptible to the current approved low concentration antibiotic discs as resistant. Data from this laboratory[3, 4] and elsewhere[1, 5, 6] have shown that staphylococci tend to be either "susceptible" or "resistant" with few in the intermediate or "moderately susceptible" (or resistant) class. When this does occur, and a note of this is to be recorded, it may be done by adding a question mark after the number of the antibiotic to which the staphylococcus shows some resistance. The fractional notation previously described may also be added.[4]

The notation SC?35KV7 on a data sheet or in a table for publication may, at first, seem too cryptic to the reader, but it requires less space and explanation than writing "only moderately susceptible to penicillin, resistant to erythromycin, tetracycline, and vancomycin, susceptible to kanamycin, but not tested with either streptomycin or chloramphenicol," especially in long tables of data on many organisms.

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* This is a portion of the paper, illustrated with slides, presented before the 27th Annual Convention of ASMT, Phoenix, Arizona, June, 1959.

A SIMPLE DEVICE FOR TRANSMISSION DENSITOMETRY*

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A simple, inexpensive attachment has been designed for the Beckman B Model Spectrophotometer which permits this instrument to be used for transmission densitometry. The device has been used successfully in the quantitative determination of proteins separated by starch-gel electrophoresis according to the method of Smithies (1), and is also applicable to paper electrophoresis and to paper chromatography.

The densitometer attachment, illustrated in Figures 1 and 2, is interchangeable with the standard cuvette rack with which the spectrophotometer is equipped.

The test sample, consisting of a transparent gel or paper strip (or photographic negatives thereof) is held horizontally on panel *A* by means of clips mounted on the inside of the panel. The aperture *B* in the panel can be adjusted for the width of the sample (within the limits of the light entrance slit in the spectrophotometer) by means of two set screws. The absorption of light by pigments in the gel or paper strips is measured as in the standard procedure for substances in solution by light transmission to the photocell.

The sample holder is moved by means of a worm drive (shown in Fig. 2) manually operated by the dial. A gear ratio of 10:1 was arbitrarily chosen permitting galvanometer readings to be taken at 1.0 mm distances along the sample by rotating the dial through a perimeter of 1 cm.

A baseline is obtained from galvanometer readings taken at the ends of the sample or from a sample-free strip parallel with it. The plate-holder can be modified to allow vertical manipulation so that two (or more) samples may be scanned simultaneously, or so that a baseline may be plotted for each setting of the dial.

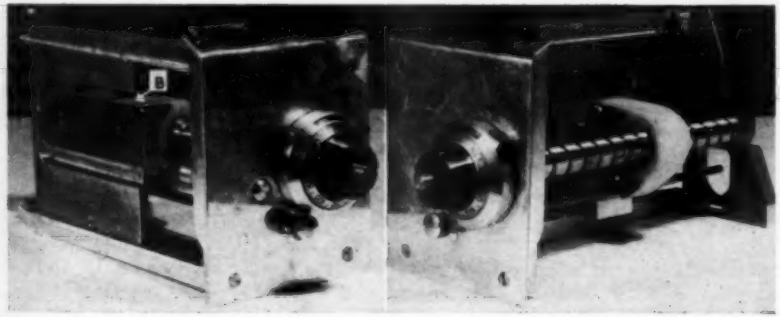

Fig. 1 and 2. Left and right views, respectively, of the densitometer attachment for the Beckman B Model Spectrophotometer. A—plate holder: the starch gel or paper strip is mounted on the inside of the plate by means of clips. B—light slot which can be adjusted by two set screws for the width of the sample.

* Received for publication June 1959.

To illustrate the results obtainable with the densitometer attachment the light absorption curve for human serum proteins separated by electrophoresis on starch gel is shown in figure 3. After staining the proteins with amido black, the opaque gel strip was cleared by heating in glycerol and light absorption through the pigmented protein bands was measured at a wave-length of 530 μ.

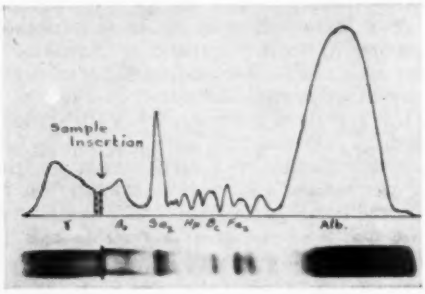

Fig. 3. Density curve obtained by transmission densitometry at 530 μ from the starch-gel pherogram of normal human serum proteins. Transparency of the gel was achieved by heating in glycerol.

Summary

A simple device is illustrated which permits the Beckman B Model Spectrophotometer to be used for quantitative determination of substances on starch gel or paper strips by transmission densitometry.

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STUDIES WITH A SINGLE STRIP TEST FOR THE SIMULTANEOUS DETERMINATION OF GLYCOSURIA, PROTEINURIA, AND URINARY pH*

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INTRODUCTION

Almost every routine urinalysis includes tests for sugar, protein, and pH[3]. Until quite recently 10 minutes or more has been required to carry out these tests on one urine sample. In the past both sugar tests and protein tests have been done by technics which involved heating over a Bunsen flame. Protein tests (or albumin tests) required clear samples of urine which in most instances are available only after centrifugation. Until recently the determination of urinary pH while not time-consuming was ordinarily reported only as being acid or alkaline to litmus paper. Significant improvements in procedures for urinalysis have been described by our laboratory during the past several years[2,4,5,6]. Such advancements have not in any way replaced the medical technologists but have made it possible for them to devote their skills and talents to the ever appearing new and complicated procedures which evolve from medical research. The present report describes studies which have been carried out with a new combination stick for simultaneous testing of urine for glucose, protein, and pH. This report provides an indication of the utility and reliability of this combination stick test called Combistix.†

THE NATURE OF THE COMBINATION STICK TEST

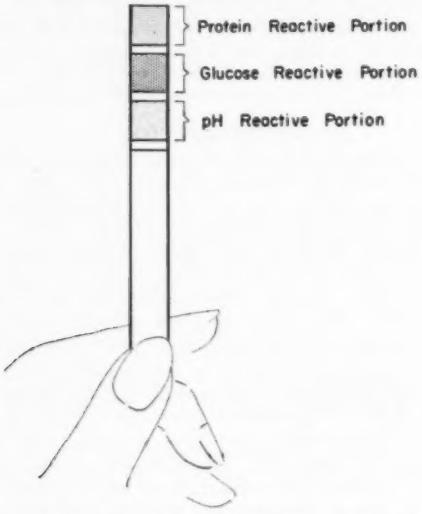
The combination stick reagent composition is composed of a piece of stiff cellulose having dimensions of approximately 75 mm. by 5 mm. It contains three reactive areas, one for protein, one for glucose and one for pH. The three areas are separated by water-impervious barriers so that the reagents in one area do not become mixed with those in another area. Figure 1 shows a diagram of the combination stick.

The Protein Reactive Portion. The ingredients used to impregnate the protein reactive portion consist of the indicator tetrabromphenol blue and a citrate buffer of approximately pH 3. Tetrabromphenol blue exhibits the "protein error of indicators" which involves the fact that at a fixed pH the indicator will have one color in the presence of protein and another color in the absence of protein. At the pH supplied by the buffer the indicator has a yellow color whereas at this same pH in the presence of increasing amounts of protein the indicator will have a green to blue color.

The Glucose Reactive Portion. The glucose reactive portion of the stick is impregnated with the enzyme glucose oxidase, the enzyme peroxidase, orthotolidine and a red dye. The oxidation of glucose by hydrogen removal is catalyzed by glucose oxidase. In this reaction gluconic acid and hydrogen peroxide are produced. The oxidation of orthotolidine by hydrogen peroxide is catalyzed by the enzyme peroxidase

* Read before the 27th Annual Convention of ASMT, Phoenix, Arizona, June, 1959.
† Registered trademark of the Ames Co., Inc., Elkhart, Ind.

Figure 1. Diagram of Combination Stick Test.



with a blue oxidation product of orthotolidine being produced. This color admixed with the red dye produces a purple color. It has been shown that this series of reactions has a very high specificity for glucose.[1]

The pH Reactive Portion. The pH reactive portion of the stick is impregnated with a mixture of methyl red and bromthymol blue These indicators were selected in order to provide a bright series of color reactions over the urinary pH range from 5 to 9. The colors are orange for pH 5, orange-yellow for pH 6, yellow-green for pH 7, dark green for pH 8 and dark blue for pH 9.

Directions for Use. The directions for the use of the combination stick test are as follows: Moisten test end of strip in urine by dipping and removing immediately. Touch tip of strip against edge of container to remove excess urine. Compare glucose portion of stick with color guide for glucose at 10 seconds. Compare the protein and pH portions with the corresponding color chart either before or after reading the glucose portion. The time of reading the pH and protein portion is not critical but it is essential that the glucose reading be made at 10 seconds after wetting. The avoidance of having excess urine on the stick eliminates any problem of urine running from one area to another over the top of the barriers.

EXPERIMENTAL DESIGN

Plan of Study. Experiments were carried out to obtain data on three different types of urine specimens for use in evaluation of results with the combination stick test and comparable single test sticks. The three different types of urine specimens consisted of: I. A series of 532 urines from hospital patients; II. A series of 109 urines from healthy subjects;

III. A series of negative urines with glucose added (174 specimens) and/or protein added (149 specimens). The combination stick test was used by both experienced and inexperienced operators to test all specimens. Their results were then compared with results obtained by experienced operators using established single test methods. Clinistix* was the comparative test used for the glucose portion, Albustix* for the protein portion, and electrometric measurement for the pH portion. Each operator used combination sticks to test a coded series of urines consisting of a random sampling of hospital urines, urines from healthy subjects, and negative urines with added glucose and protein. The source of each urine specimen was unknown to the operator at the time of testing. Some of the inexperienced operators were using the combination stick test for the first time and some of these operators were not even laboratory workers. Each unknown series was tested by approximately six different operators.

Single Test Methods Employed. The comparative single test methods were as follows:

1. Enzymatic Stick Test for Glucose—Clinistix. This is a simple dip-and-read test in which all reagents have been impregnated into a paper strip. The test is based on the oxidation of glucose by hydrogen removal catalyzed by the enzyme glucose oxidase. The reaction is quite specific for glucose[1] and will not react with nonglucose reducing substances. The test is carried out by dipping the test stick into the urine and observing the color of the stick 1 minute after removing it from the specimen. The stick will remain colorless in the absence of glucose and will turn light, medium, or dark blue in the presence of glucose.

2. Colorimetric Protein Stick Test—Albustix. This is a colorimetric dip-and-read test in which all reagents have been impregnated into a paper strip. The test is based on the "protein error of indicators" and has a composition comparable to that of the protein reactive portion of the combination stick test. The strip is dipped into a urine specimen and compared with a color chart having color blocks representing, 0, 30, 100, 300, and 1000 mg.% protein. If protein is absent, the reaction remains yellow. If protein is present, the color may range from yellow-green through green to blue depending upon the quantity of protein present.

3. Electrometric pH Determination. A Leeds and Northrup battery operated pH meter was used for measuring the pH of the urines. This is a precise instrument and is a far more exact means of measuring urinary pH than procedures ordinarily used in routine urinalysis. With this instrument readings were made to the nearest 0.1 of a pH unit.

RESULTS

In Series I (urines from hospitalized patients) replicate tests by experienced and inexperienced operators resulted in the use of 2719 combination sticks to test 532 urines for protein, pH, and glucose. In Series II (urines from presumably healthy subjects) 580 combination sticks were used to test 109 urines for protein, pH, and glucose. In Series III, 959 combination stick results were obtained on the 174 specimens containing added glucose and 798 combination stick results were obtained

* Clinistix and Albustix are registered trademarks of the Ames Co., Inc., Elkhart, Indiana.

on the 149 specimens containing added protein. Some specimens contained added glucose only, some contained added protein only, and some contained both added glucose and added protein. Results on urines containing glucose only were omitted from protein tabulations since they did not contain added protein. Similarly urines containing added protein only were omitted from the glucose tabulations since they did not contain added glucose. Specimens containing both glucose and protein appear in both tabulations. The pH of the urines was not affected by glucose or protein additions; therefore, the pH values obtained with combination sticks in Series III are not presented in this report.

Glucose Portion of the Combination Stick Test. Table I shows glucose results obtained by inexperienced and experienced operators who were using the combination sticks. All urines, hospital or normal, which were negative with Clinistix were also negative with the combination stick test. In the normal series, all urines which were positive with Clinistix were also reported as positive with the combination stick test. In the hospital series, urines which were positive with Clinistix were reported as negative 15 per cent of the time with the combination stick. This group of urines gave very light reactions with Clinistix at the one minute reading time. With minute amounts of glucose, color may not be present at 10 seconds but can definitely be seen as a light shade at 1 minute. The shorter reading time of the combination stick test tends to decrease somewhat the sensitivity of this enzyme test. However as noted in the data from Series III, the combination test can still detect the addition of 0.1% glucose in 90 per cent of the tests. Therefore even

TABLE I

Results with Glucose Portion of Combination Stick Test

		COMBINATION STICK TEST	
		Reaction	No. of Tests
Series I 532 Random Urines from Hospital Patients	Negative (Clinistix)	100% Negative	2426
	Positive (Clinistix)	15% Negative* 85% Positive	293
Serie II 109 Urines from Healthy Subjects	Negative (Clinistix)	100% Negative	565
	Positive (Clinistix)	100% Positive	15
Series III 174 Specimens with Added Glucose	0.1% added	10% Negative 62% Light 24% Medium 4% Dark	184
	0.25% added	13% Light 43% Medium 44% Dark	181
	0.5% added	3% Light 22% Medium 75% Dark	134
	1%—4% added	2% Light 16% Medium 82% Dark	460

* With minute amounts of glucose, reactions which are negative at a ten-second reading time (combination stick test) may be light blue at a one-minute reading time.

with a 10 second reading time, it is still an extremely sensitive test for glucose.

Protein Portion of the Combination Stick Test. Data on the protein tests are presented in Table II. Reactions for naturally occurring protein in urines from presumably normal people or in urines from hospitalized patients have been grouped in this table as negative, as trace, or as positive (30 mg.% or more). The table indicates that there is good correlation between single stick results obtained by experienced operators and combination stick test results obtained by inexperienced as well as experienced operators. In experiments where protein was added to negative urines, there is good correlation between the amount of protein added and the amount reported by people using the combination stick test.

TABLE II

Results with Protein Portion of Combination Stick Test

		COMBINATION STICK TEST	
		Reaction	No. of Tests
Series I 532 Random Urines from Hospital Patients	Negative (Albustix)	92% Negative 8% Trace	1646
	Trace (Albustix)	39% Negative 49% Trace 12% Positive	607
	Positive (Albustix)	18% Trace 82% Positive	466
Series II 109 Urines from Healthy Subjects	Negative (Albustix)	90% Negative 10% Trace	538
	Trace (Albustix)	61% Negative 29% Trace 10% Positive	31
	Positive (Albustix)	27% Trace 73% Positive	11
Series III 149 Specimens with Added Protein	10 mg. % added	26% Negative 56% Trace 18% 30 mg.%	242
	30 mg.% added	1% Negative 8% Trace 86% 30 mg.% 5% 100 mg.%	256
	100 mg.% added	1% Trace 3% 30 mg.% 77% 100 mg.% 19% 300 mg%	132
	300 mg.% added	92% 300 mg.% 8% 1000 mg.%	98
	1000 mg.% added	31% 300 mg.% 69% 1000 mg.%	70

pH Portion of the Combination Stick Test. Table III correlates pH results on the combination stick with pH results obtained by electrometric determinations. The color chart for pH which accompanies the combination stick test has a color block for each whole pH unit and stick values therefore were reported only in whole pH units. The electrometric values were read and reported as the closest 0.1 pH unit. Urines with electrometric pH values between 5.5 and 6.5 should match

the pH 6 color block for the combination stick test. Similar ranges of ±0.5 unit should be expected with data for the other pH color blocks. Data from urines in Series I and II shows that at each pH level this ideal accuracy was attained from 64 per cent to 100 per cent of the time. In all the remaining cases the pH value which was reported represented the pH of the adjacent color block. The pH values obtained with the combination stick test showed good correlation with the electrometric determinations.

TABLE III

Results with pH Portion of Combination Stick Test

	Meter pH Range	COMBINATION STICK TEST	
		Reaction	No. of Tests
Series I 532 Random Urines from Hospital Patients	4.9—5.5	94% pH 5 6% pH 6	669
	5.6—6.5	24% pH 5 75% pH 6 1% pH 7	1168
	6.6—7.5	18% pH 6 71% pH 7 11% pH 8	572
	7.6—8.5	2% pH 7 82% pH 8 16% pH 9	215
	8.6—9.1	100% pH 9	95
Series II 109 Urines from Healthy Subjects	4.8—5.5	100% pH 5	185
	5.6—6.5	32% pH 5 67% pH 6 1% pH 7	238
	6.6—7.5	21% pH 6 64% pH 7 15% pH 8	135
	7.6—7.8	27% pH 7 73% pH 8	22

DISCUSSION

The data which have been presented in this paper demonstrate that the combination stick test is a convenient means for the rapid testing of urine for glucose, protein, and pH. Only a little more than 10 seconds is required to test a single urine specimen for all three constituents. There is no need for any preliminary treatment of the urine specimen and there is only a single paper strip to dispose of afterward. The data presented in this paper indicate that the combination strip test could be a useful adjunct in the performance of routine hospital urinalyses. It is also a reliable means for the preliminary screening of large numbers of urines from presumably healthy people. Data indicate that the glucose and protein test portions have adequate sensitivity for either purpose and that the pH portion is an accurate means of estimating the pH of all these urines.

The simplicity of the test is such that satisfactory results can be obtained regardless of experience or inexperience of the person using the combination stick test. This may be a feature of great importance in

many laboratories because of the shortage of trained personnel. The combination stick test facilitates the use of ancillary personnel and allows trained technologists to spend more time in carrying out the newer and more complicated clinical laboratory procedures.

SUMMARY

An evaluation study was carried out with a simple combination stick test. The test stick is a single piece of stiff bibulous cellulose with separate reaction areas for glucose, protein, and pH. In approximately a ten second period it is possible to test for all three constituents in one simple dip-and-read operation. Data from studies on coded urine specimens demonstrate that both trained and untrained operators obtain reliable results with the combination stick test (Combistix). The ease of testing simultaneously for three urinary constituents enhances the utility of the test in routine laboratory operations and in mass screening programs.

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CORRECTION

In the July-August 1959 issue page 250, the correct spelling for the co-author's name is Edward KRAVITZ.

A RAPID CHEMICAL ASSAY FOR TOTAL URINARY CATECHOLAMINES†

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Reliable chemical methods for urinary levels of adrenalin and noradrenalin are now well established.[1,4] In order to simplify these methods and make them available to the laboratories of hospitals and small clinics, Hingerty[2] devised a technique eliminating column chromatography and reading the results by visual inspection. The method described in this paper uses Hingerty's basic technique but with adaption to the Coleman Photofluorometer in order to improve on the reliability and reproducibility of the analysis. Quinine sulfate is used as a control on all fluorescence developed. A single assay takes about thirty minutes.

Method

Specimen

24 hour urine sample has been used to establish normal values and this specimen is commonly used. It is collected without preservative but kept under refrigeration. If the patient appears clinically acute any specimen of approximately 100 ml. may be used.

Reagents

1. Aluminum oxide (Baker and Adamson Reagent Grade ignited powder has proven satisfactory). Place 200 gm. of this powder in a flask, add a liter of N MCl and bring to a boil. Allow to settle, pour off the excess acid and wash several times with distilled water. The last washings can be made on a Buchner funnel. Dry the powder at 110° C. and store at room temperature.
2. 1% phenolphthalein in 95% ethanol.
3. 20% sodium hydroxide.
4. N/5 sulfuric acid.
5. 2% sodium bicarbonate.
6. 0.25% potassium ferricyanide.
7. 2% ascorbic acid. Stable 48 hours at refrigerator temperature.
8. Alkaline—ascorbic acid solution. Add 0.5 ml. of 2% ascorbic acid to 4.5 ml. of 20% NaOH. Make fresh for each analysis.
9. Stock Standard Noradrenalin: Weigh 200 mg. of Levophed* bitartrate monohydrate into a 100 ml. volumetric flask. Dissolve in isotonic saline and add 200 mg. of sodium bisulfite as a preservative. Dilute to the mark with saline. Each ml. of this solution represents 1 mg. of Noradrenalin base.
10. Working standard. Dilute 1 ml. of the stock to 100 ml. with distilled water. This represents 10 mcg. per ml. and is stable 48 hrs. at refrigerator temperature.

Procedure

1. Measure the urine volume and transfer 100 ml. into a 400 ml. beaker marked X.

† Received for Publication April 1959.

* Levophed—our stock was donated by Sterling-Winthrop Research Institute, Rensselaer, N. Y.

2. Place 100 ml. of a control urine (any normal voided specimen) in another 400 ml. beaker marked S.
3. Add 100 ml. of distilled water to beaker X and 95 ml. to beaker S.
4. Add 5 ml. (50 mcg.) of the Working Standard to S.
5. Add 2 gm. of the aluminum oxide to each beaker.
6. Adjust each solution to pH 8.5 using phenolphthalein and 20% NaOH.
7. Continue to stir for 1 minute to insure complete absorption of the catechols.
8. Allow to settle and pour off the supernatant (a fine precipitate of phosphates will remain suspended but is not concerned with the test and should be discarded.)
9. Add about 200 ml. of distilled water to each beaker. Stir again for about 1 minute, settle and pour off supernatant as before.
10. Carefully transfer the precipitate to a 15 ml. centrifuge tube using a wash bottle.
11. Balance the tubes with water and centrifuge for 2 minutes at medium speed.
12. Invert to drain water without losing any aluminum oxide.
13. Add 5 ml. of N/5 H_2SO_4 to each tube and stir for 1 minute with a glass rod.
14. Centrifuge for 2 minutes at medium speed.
15. Place 3 ml. of the acid eluate from X in a test tube marked X. Place 1 ml. of the eluate from S in a test tube marked S. Add 2 ml. of N/5 H_2SO_4 to the tube marked S.
16. Add 3 ml. of 2% $NaHCO_3$ to each tube, tap to mix and shake, releasing the CO_2 pressure carefully and frequently until there is no further reaction.
17. Add 0.5 ml. of 0.25% $K_3Fe(CN)_6$ to each tube, mix and allow to stand 2 minutes.
18. Add 1 ml. of fresh alkaline ascorbic acid mixture to each tube.
19. Mix and centrifuge at medium speed for 2 minutes.
20. Pour off into Photofluorometer Cuvettes marked "S" and "X."
21. Prepare another Photofluorometer Cuvette with distilled water to serve as a blank "B."
22. The sample is read in the Coleman Photofluorometer (Model 12 C) using Corning Filter No. 5970 (primary) and Corning Filter No. 3385 (secondary).
23. The aperture is set at 90 with tube S. The zero is set with tube B, and the sample (X) is read directly from the scale. This result is multiplied by 2 to obtain mcg/liter of urine.

Calculations

1. The 100 ml. of control urine is expected to contain about 4 mcg. of catecholamines. 50 mcg. are added (step 4) to make a total of 54 mcg. or 540 mcg. per liter. This extract is then diluted 1 to 3 (step 15) to represent a final concentration of 540/3—180 mcg. per liter. The instrument is set at 90 for convenience since the scale only reads to 100.

2. The concentration value of the total urine catechols, obtained in Step 23, is multiplied by the number of liters of urine in the 24 hour specimen to obtain the excretion in mcg./24 hrs. Normal values by this procedure range 15-140 mcg/24 hr.

Results

A chromaffin cell tumor of the adrenal medulla will increase the level of urinary catecholamines 10-100 times normal. With this method, values as high as 2900 mcg/24 hours have been observed in our laboratory. Since this condition is relatively rare, the sensitivity of the method was tested by determining increases of total urinary catecholamines in college football players before and after practice sessions.

The results indicated in Table 1 show the method to be sensitive enough to determine increases as small as 20 mcg./liter. These specimens were from five different practice sessions. (*See Table 1*).

With the use of the photofluorometer rather than the visual comparison technique of Hingerty,[2] it is possible to carry through a quinine sulfate standard as a reagent control. We have prepared a standard containing 0.1 mcg. of quinine sulfate per ml. in N/10 sulfuric acid which gives a 64/90 fluorescence ratio with noradrenalin reading 90. This material is not processed through the procedure but acts only as a direct check on the standard when it is read.

TABLE 1

Total catecholamines on football players before and after practice sessions (all results in mcg/liter)

PLAYER	Before	After	Increase
1.	40	94	54
2.	66	104	38
3.	64	184	120
4.	104	124	20
5.	84	108	24
6.	88	110	22

SUMMARY

Using the relatively inexpensive Coleman Photofluorometer and the simplified method of Hingerty, total urinary catecholamines can be determined in less than 30 minutes. Tests run on football players demonstrate increments varying from 20 to 120 mcg/liter during practice sessions. A 0.1 mcg/ml. quinine sulfate standard is used as a reagent control.

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ACKNOWLEDGMENTS

We wish to thank Coach Jim Malosky and the members of the football team at the University of Minnesota (Duluth Branch) who participated in this study.

BETTER PARAFFIN SECTIONS WITH THE AID OF VACUUM*

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The value of the use of vacuum in processing tissues has been recognized for about a decade. When used in fixation, processing time is reduced and more complete penetration is accomplished.[1] Again, use of the vacuum to de-gas the tissue during infiltration gives better impregnation with paraffin. Control of the pressure with a standard vacuum oven has proved to be satisfactory when using this procedure on whole organs or large pieces of bone.

Adapting this principle with the use of a simple, inexpensive unit similar to that described by Weiner,[2] the authors have found it possible to produce better sections of a variety of tissues, and they can be cut with less difficulty than those that are handled by conventional methods. The use of vacuum has, therefore, been adopted by this installation as a routine step in the processing of all types of tissues, including human and animal eyes, bone, lung, nerve, and other soft tissue.

The vacuum head consists of a one-half inch plastic disc about 6 inches in diameter wth a central opening. On the under side of this disc is a rubber ring three-sixteenths of an inch thick and seven-eighths of an inch wide, placed flush with the outer edge of the disc, which acts as a gasket. Through the central opening is a brass cross joint, at each end of which is a gas stopcock. To one is attached the hose from the vacuum or motor pump, while the other serves as an air valve. This assembly can be used on any metal container of a size similar to a paraffin pot, which is standard equipment on most of the various tissue processors.** (See Figure 1.)

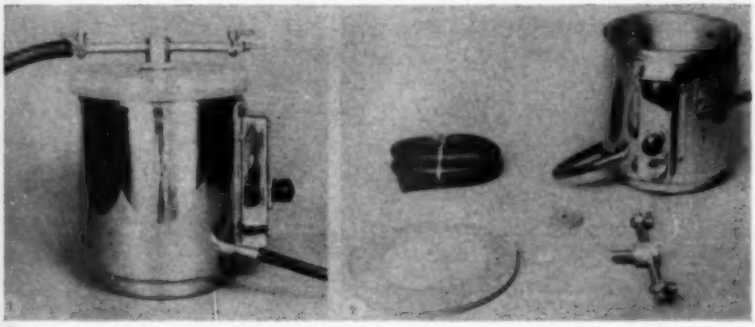

Figure 1. (AFIP 59-11751). Paraffin pot with head for vacuum processing.
1. Unit assembled for use.
2. Various components of unassembled unit.

* Received for publication June 1959.
** Acknowledgment is given to Mr. R. F. Davis, Chief, Construction Branch, Scientific Illustration Division, Armed Forces Institute of Pathology.

Following the two paraffin baths usually employed by most technics, the basket of tissue is placed in a third paraffin bath, to which is attached the vacuum head described. Application of a moderate, even pressure is made and kept constant for about 1 hour. Tissue is then embedded in the usual manner.

After use of the vacuum, the tissue is found to be free of tiny air holes or bubbles frequently encountered in other embedded tissue. This reduces the tendency of the tissue to adhere to the cutting edge of the knife, to pull or tear as it contacts the knife edge through such holes, and to be only partially infiltrated with paraffin. (See Figure 2.)

When "rush" reports are required the same day on which the material is received, it is possible to use the vacuum head to advantage while processing the tissue by hand. It speeds up both dehydration and im-

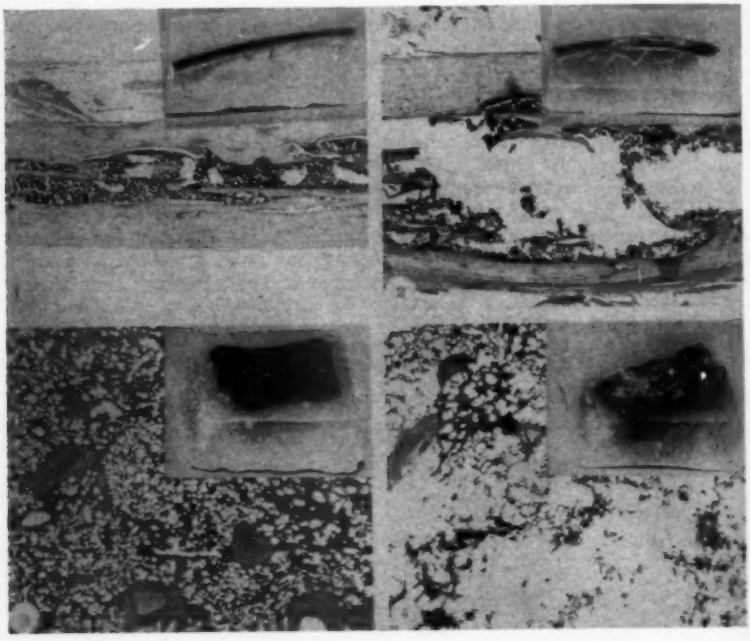

Figure 2. (AFIP 59-11751). Completed slides and paraffin blocks processed with and without use of unit.

1. **Hematoxylin and eosin stain on section (x14) cut from paraffin block of bone (insert) after infiltration with aid of vacuum head.**
2. **Hematoxylin and eosin stain on section (x14) cut from paraffin block of bone (insert) after processing without use of vacuum.**
3. **Hematoxylin and eosin stain on section cut from paraffin block of lung (x9) (insert) after infiltration with aid of vacuum head.**
4. **Hematoxylin and eosin stain of section cut from paraffin block of lung (x9) (insert) after processing without use of vacuum.**

pregnation. (Because of the deleterious effect of most clearing agents on both the rubber gasket and the plastic disc, the use of the vacuum head during the step of clearing is omitted.) The seeming loss of time at this point is compensated by the value of its use during the dehydration and impregnation steps respectively.

Summary

This installation has adapted a unit that it uses routinely to de-gas various kinds of tissue by means of vacuum during infiltration. The use of this unit as a regular step in processing accomplishes the following:

1. Reduces the total time of processing.
2. Gives more complete impregnation.
3. Eliminates distortion of tissues that results from precutting the gross specimen for processing in order to get complete infiltration.
4. Produces smoother sections without tears or holes.
5. Reduces time for sectioning blocked material.

The choice of plastic over glass for the disc has been made on the basis of its strength and durability, its lower cost, and the ease with which it can be fabricated.

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LABORATORY SUGGESTION

BACTERIOLOGY; STORAGE OF MEDIA

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Often it is necessary to store prepared petri dishes over some length of time. One of the biggest problems encountered in such storage is drying of the media in those plates which are kept for a long period of time. In fact our experience indicated that two weeks storage was an extreme limit before drying of the media made the plates worthless. This is of particular importance in a laboratory where prepared plates are not furnished but, rather, one must use dehydrated media to prepare culture plates.

We have found a way to overcome this difficulty. The method is both effective and inexpensive. We have placed prepared plates in the plastic bags which laundries use to protect men's shirts, rolled the open end of the plastic bag, and fastened it with a spring clamp. Prepared plates have been kept in this manner for as long as six months, and when used were found to have moist surfaces and to show no drying effects whatsoever.

A REPORT OF THE OCCURRENCE OF MAST CELLS IN ATROPHIC TYPE VAGINAL AND CERVICAL SMEARS†

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THOMAS R. TURNER, *Master Sergeant, Army Medical Service***

The presence of single unusual cells which frequently contain deep basophilic nuclei has been observed by us in 4.3 per cent of 1475 cases of atrophic type vaginal and cervical smears. These cells may be interpreted by inexperienced personnel as Class III and occasionally Class IV type cells. We consider these particular cells to be benign. In our study, they have been noted only in the smears of exfoliated cells harvested from menopausal women.

These cells are troublesome by virtue of their infrequent appearance in vaginal and cervical smears. Fortunately, they fail to satisfy the cytological criteria for malignancy. These cells are oval in configuration. They contain a round vesicular nucleus or a deep basophilic staining dense nucleus. Rarely, two nuclei are present. Nucleoli are quite infrequent. The cytoplasm is packed with numerous, basophilic granules which are equal in size and may partially obscure the nucleus. Study of Figures 1, 2 and 3 permits a comparison of these cells with a typical tissue mast cell. Histochemical staining of these cells demonstrates the metachromatic staining character of the intracytoplasmic granules when exposed to toluidine blue stain. Our objectives are to describe the cell in detail, point out its remarkable resemblance to tissue mast cells, and establish its benign character.

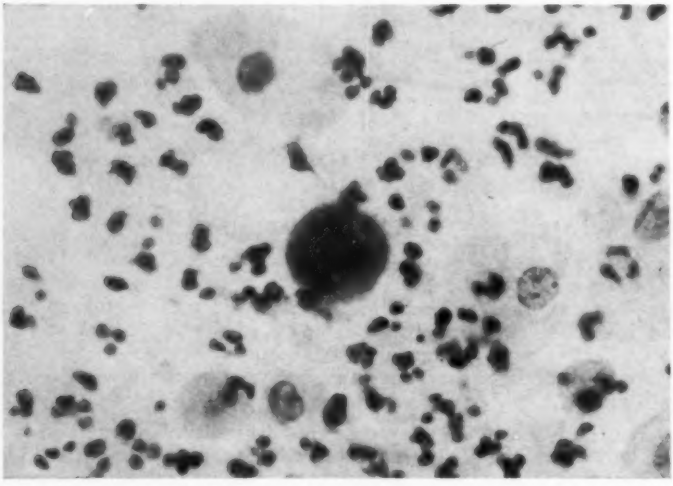

Fig. 1. Mast Cell in Vaginal Smear, Papanicolaou Stain; Reduced from X610. (U. S. Army Photograph.)

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** Cyto-technologist (ASCP), Laboratory Service, Walter Reed Army Hospital, Walter Reed Army Medical Center, Washington, D. C.
† Received for publication June 1959.

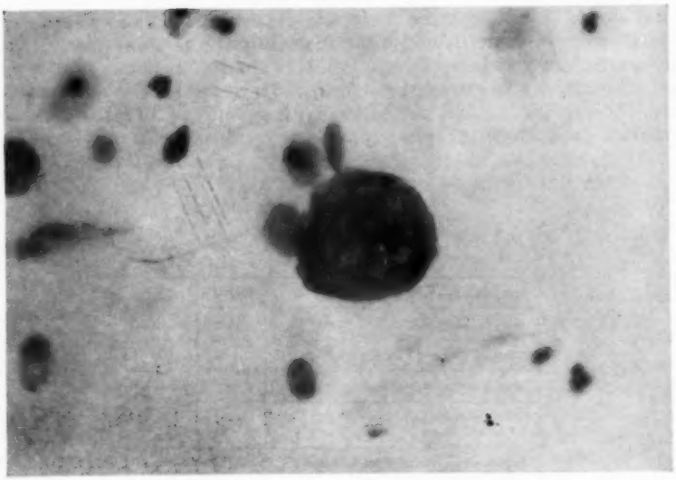

Fig. 2. Mast Cell Containing Two Nuclei, Papanicolaou Stain; Reduced from X610. (U. S. Army Photograph.)

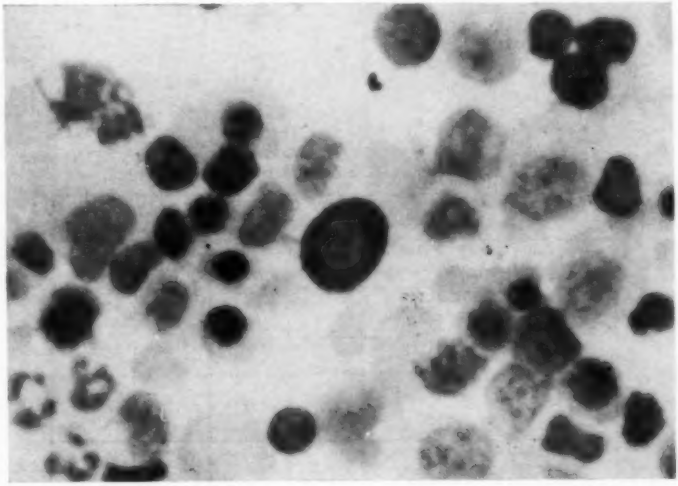

Fig. 3. Tissue Mast Cell in Bone Marrow, Wright's Stain; Reduced from X610. (U. S. Army Photograph.)

We reviewed all female genital cytology cases from 1954 through 1957. A total of 1475 cases were atrophic-type smears. All of these were analyzed with respect to cell type distribution, trichomonads, bacteria, yeast, granulocytes, histiocytes, cellular debris, mucus, and hormonal effect. The salient features are listed in Tables I and II. We have arbitrarily chosen the term "atrophic changes" to denote the presence of these mast cells in an atrophic-type smear. These cells may occur singly

STATISTICS

	Number of Cases	Percentage of Cases
Total Cases Reviewed (1954-1957)	27,700	---
Atrophic Type Smears	1,475	5.3 %
"Atrophic Changes" (42 Patients)	64	4.3 %
Patients with No Repeat Smear	17	40.0 %
Patients with "Atrophic Changes" on Repeated Smears	13	30.0 %
Patients with a History of Total Abdominal Hysterectomy-Bilateral Salpingo-Oophorectomy or Supra-Vaginal Hysterectomy	22	52.0 %
a. Reasons Unknown	12	29.0 %
b. Malignant Diagnosis	10	24.0 %
Patients with a Subsequent Malignant Diagnosis	0	---
Average Age of Patients	60 Years	
Average Time "Atrophic Changes" Disappeared	8.1 Months	

TABLE I

ATROPHIC TYPE SMEARS
SIGNIFICANT CELLULAR AND PARASITIC DISTRIBUTION

	Trichomonas		Parabasal Cells		Cornified Parabasal Cells	
Distribution	With Atrophic Changes	Without Atrophic Changes	With Atrophic Changes	Without Atrophic Changes	With Atrophic Changes	Without Atrophic Changes
None Seen To A Few	6.27%	33.39%	1.56%	28.59%	14.05%	50.81%
Moderate To Numerous	24.99%	33.33%	42.09%	61.89%	56.24%	39.67%
Innumerable To Extremely Heavy	68.74%	33.28%	56.35%	9.52%	29.71%	9.52%

TABLE II

or in small groups scattered throughout the smear. Their presence correlates with low hormonal effect, increased numbers of slightly reactive parabasal and cornified parabasal cells, increased numbers of trichomonads, and to a lesser extent, granulocytes, cellular debris, and fine threads of basophilic mucus (Figure 4). When the smears were repeated, the mast cells proved to be transient, occurring in 30 per cent of 18 repeated smears. In none of the studied cases did subsequent malignancy develop. Ten patients had a previous diagnosis of malignancy, but none showed evidence of recurrence. Twelve had a previous recent history of surgery for benign conditions.

Discussion

Mast cells have not been reported to occur in exfoliated cell smears of the human female genital tract. Mast cells, however, have been observed in the mouse vagina and uterus by Johansson and Westin,[1] and Westin and Odeblad.[2] These investigators have studied the effects of varying levels of testosterone or estrogen on the quantitative numbers of tissue mast cells by vaginal smear and tissue examination. They recognized an inverse relationship between the total number of mast cells and the level of sex hormone used. It has been suggested by these investigators that the decrease of mast cells with disappearance of their metachromatic cytoplasmic granules may be related to the growth stimulus induced by testosterone or estrogen administration. Padawer[3] reviews the physiology of tissue mast cells and, among other observations, states that their cytoplasm contains various amounts of heparin,

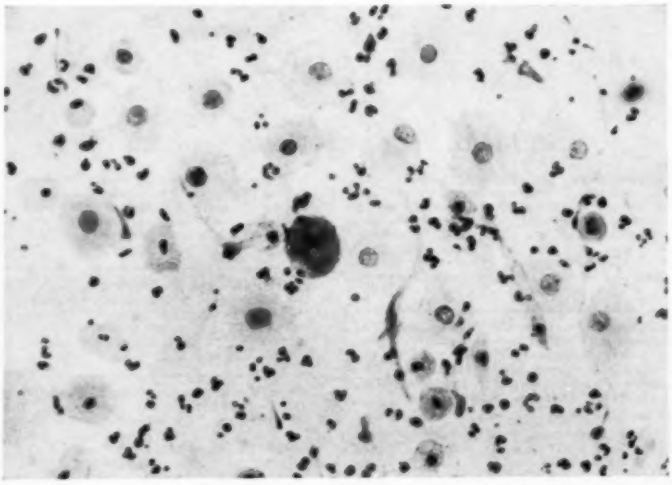

Fig. 4. Parabasal and Cornified Parabasal Cells, Mast Cell, Granulocytes and Trichomonads Papanicolaou Stain; Low Power. Reduced from X610. (U. S. Army Photograph.)

histamine, and serotonin. Despite these detailed studies, the actual physiological role of tissue mast cells remains unknown.

We postulate that mast cells may appear infrequently in the pool of exfoliated cells during the atrophic phase of reduced estrogen activity and possibly following periods of growth or active repair of the local tissues. It is during this period of time that the mast cells may be discovered in the vaginal or cervical smear. Unfamiliarity with this benign cell may lead to erroneous classifications and undue patient and physician concern.

Summary

Benign cells having a remarkable resemblance to tissue mast cells were observed in 4.3 per cent of 1475 cases of atrophic-type vaginal and cervical smears. These mast cells may be misinterpreted by inexperienced personnel. Their presence appears to be inversely related to the level of estrogens.

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MEDICAL TECHNOLOGISTS WANTED

Medical Technologists, Registered (ASCP) or eligible for progressive teaching hospital. 40 hour week with paid vacation and sick leave. Salary commensurate with education and experience. Increases given according to merit. No night calls. Interested parties contact: Mildred M. Tucker, Technical Supervisor, Mount Sinai Hospital, 2750 W. 15th Place, Chicago 8, Illinois.

New Hospital Laboratory in north central Texas offers challenging opportunities to qualified (ASCP or eligible) medical technologists; interesting work with specialist staff; many employee benefits; salary open. Administrator, Bethania Hospital, Wichita Falls, Texas.

Histologic Technologist: College graduate with ASCP registration preferred. At least five years experience in tissue work. Salary commensurate with education and experience plus usual prerequisites. Academic environment and stimulating work—many personnel benefits—excellent living conditions—sunshine and 30 miles of sandy beach on the Gulf of Mexico—mild winters. Reply: Department of Pathology, University of Texas—Medical Branch, Galveston, Texas.

Wanted: Medical Technologist (ASCP or eligible) for accredited hospital in laboratory staffed by certified pathologist, two other registered technologists and four technicians. Contact: Administrator, Lewistown Hospital, Lewistown, Pennsylvania.

Medical Technologist—Registered ASCP or eligible. 316 bed general hospital. 30 full time pathologists—approved school for medical technology. No night call, 40 hour week. Starting salary based on experience and education. Fine suburban community 20 miles from New York City. Apply Personnel Office, Overlook Hospital, Summit, New Jersey.

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